BELOWGROUND CONTRIBUTIONS OF PEA AND CANOLA TO SOIL NITROGEN POOLS AND PROCESSES

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ABSTRACT

Nitrogen (N) contained in roots and rhizodeposits represents a significant input of crop residue-N into soil that is often unaccounted, despite its contribution to the total N budget and its influence on soil nutrient cycling. Utilizing ^{15}N-labeling methodologies under controlled conditions, the goal of this research was to quantify the input of belowground N (BGN), including rhizodeposits and roots, to soil and to investigate the influence of BGN on soil N cycling processes from the major pulse and oilseed crop grown across the Canadian prairies—namely, field pea and canola, respectively. Using continuous $^{15}N_2$ labeling, the input of fixed-N to rhizosphere soil from pea plants amounted to less than 2% of the total plant N assimilated via fixation. Nodulation and root ^{15}N enrichment were positively related to rhizosphere ^{15}N enrichment, suggesting that the relatively low input of fixed-N to soil was due to low N fixation in this system. Shoot ^{15}N-labeling techniques enabled a higher ^{15}N enrichment in roots; as a result, rhizodeposition was detected in the rhizosphere as well as the surrounding bulk soil. Rhizodeposition accounted for 7.6 and 67% of plant N and BGN, respectively, in mature pea. Temporal changes in the pattern of rhizodeposition were detected as evidenced by differing ^{15}N enrichment in rhizosphere versus bulk soils. In comparison to pea, a higher proportion of BGN contributed to the total residue-derived N from canola. The higher quantity of N rhizodeposition by canola was related to greater root biomass. However, pea rhizodeposition contributed more to soil inorganic N pools; this was sustained over time, as a higher proportion of pea BGN contributed to the growth of a subsequent wheat crop. In addition, wheat uptake of residue-derived N was twice as much from belowground compared to straw residues. Whereas the abundance of denitrifying bacterial communities in the rhizosphere was uncoupled from rhizodeposition and denitrification enzyme activity (DEA), root-derived ^{15}N correlated with DEA in pea and canola. This research highlights the importance of belowground inputs from differing crop species on N budgets and soil N cycling.

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LIST OF ABBREVIATIONS

AG	Aboveground
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BG	Belowground
BGN	Belowground nitrogen
BNF	Biological N_2 fixation
bp	Base pair
BS	Bulk soil
bv	Biovar
cv	Cultivar
CV	Coefficient of variation
DAS	Days after sowing
DEA	Denitrification enzyme assay
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
F	Flowering
HSD	Honestly significant difference
M	Maturity
NA	Natural abundance
nar	Nitrate reductase
NHI	Nitrogen harvest index
nir	Nitrite reductase
nirK	Gene encoding the copper containing subunit of nitrite reductase
nirS	Gene encoding the cytochrome cd1-containing subunit of nitrite reductase
nor	Nitric oxide reductase
nos	Nitrous oxide reductase
nosZ	Gene encoding a structural subunit of nitrous oxide reductase
%Ndfa	Percentage nitrogen derived from atmosphere
%NdfR	Percentage nitrogen derived from rhizodeposition

%Ndfr	Percentage nitrogen derived from residues
PF	Pod filling
qPCR	Quantitative polymerase chain reaction
RS	Rhizosphere soil
sd	Standard deviation
SIP	Stable isotope probing
SOC	Soil organic carbon
V	Vegetative

1. GENERAL INTRODUCTION

1.1. Introduction

Nitrogen (N) is a primary constituent of proteins and nucleotides and therefore is fundamental for all life, yet it is often the most limiting nutrient for crop production. In intensive agricultural systems, crop yields are sustained and increased with the addition of N fertilizer (Vitousek et al., 2002). However, crops access only a portion of this applied N (Gardner and Drinkwater, 2009), thus fertilizer applications can exceed crop uptake of fertilizer N (Drinkwater and Snapp, 2007), resulting in the buildup of excess inorganic N in the soil (Lu et al., 2011). Soil inorganic N is highly mobile and reactive, and when present in excess can have harmful consequences for the environment including contamination of ground and surface waters, production of the potent greenhouse gas, nitrous oxide (N_2O), production of N-oxide gases that contribute to air pollution, as well as soil acidification and alteration of natural ecosystems by atmospheric N deposition (Robertson and Vitousek, 2009; Vitousek et al., 1997). The processes that comprise the N cycle are mediated predominantly by microorganisms and are highly dynamic and difficult to predict. Indeed, managing the N cycle to meet crop requirements and minimize N losses from agroecosystems remains a significant challenge despite decades of research (Drinkwater and Snapp, 2007; Gardner and Drinkwater, 2009; Janzen et al., 2003; Robertson and Vitousek, 2009).

Crop type and crop rotation are important factors to consider in optimizing N management (Grant et al., 2002). In particular, increasing the level of complexity of crop rotations may represent a means to improve the overall N use efficiency of cropping systems (Robertson and Vitousek, 2009). Plant species differ in their N requirements and in the efficiency with which they access nutrients (Knops et al., 2002). For example, root characteristics, such as depth and distribution, vary among crop species (Gan et al., 2009c; Gastal and Lemaire, 2002; Liu et al., 2011), thus affecting nutrient access and uptake (Campbell et al., 2006; Kristensen and Thorup-Kristensen, 2004). Some crop species, including canola, do not form mycorrhizal associations and cannot benefit from potential increases in N access. Increasing the diversity of crop species

through rotations takes advantage of differences in N demands and acquisition strategies. Legumes are particularly important in crop rotations as they acquire a portion of their N from biological N_2 fixation (BNF) and can improve soil N supply (Campbell et al., 1992). Diverse crop rotations with reduced fallow periods can reduce soil nitrate (Campbell et al., 2006; Malhi et al., 2009). Therefore, careful selection of cropping sequences can reduce the potential for N loss as well as optimize N supply. In addition, it is important to consider crop specific effects on N cycling along with other factors such as plant disease, weed competition, and moisture availability in order to establish synergistic rather than antagonistic cropping sequences (Liebig et al., 2007).

Nitrogen is recycled primarily through the decomposition of crop residues that are returned to the soil (Lupwayi and Kennedy, 2007). Coupled with soil microbial activity, residue quality regulates the rate and pattern of N mineralization from crop residues (Lupwayi and Kennedy, 2007). In addition, the quality of crop residues can influence the fate of N fertilizer, with residues of wide C to N ratios causing the added N to be immobilized (Grant et al., 2002). Despite the influence that crop residues have on the N cycle, studies that trace the fate of residue N are much less prevalent than those that trace the fate of fertilizer N in soils. Indeed, in a meta-analysis of a global data set of the fate of added ^{15}N in temperate agroecosystems, Gardner and Drinkwater (2009) highlight that the number of ^{15}N studies that examine crop residue N are limited relative to those that examine fertilizer N. These authors noted a particular lack of data on belowground residue, which includes roots and rhizodeposits (Gardner and Drinkwater, 2009). Limited research on quantifying belowground N and its fate in the environment has contributed to an incomplete understanding of the influence that specific plant species have on N cycling. Furthermore, quantifying the total amount of N input to soil via roots and rhizodeposits provides a more complete N budget (Janzen et al., 2003), which is particularly important in evaluating the N benefits of legumes to crop rotations (Peoples et al., 2009). Designing N efficient cropping systems relies on the acquisition of more information regarding the inputs and the dynamics of N from crop residues, particularly from belowground inputs of N from both legume and non-legume crops.

The overall goal of this project was to quantify N in roots and rhizodeposits from the dominant pulse and oilseed crop grown in Prairie agroecosystems—namely, field pea and canola, respectively—and their contribution to soil N pools, plant N uptake, and N cycling processes.

This was accomplished by employing ^{15}N isotope labeling of plants under controlled conditions, using either natural or artificial modes of ^{15}N assimilation.

1.2. Organization of the Dissertation

The research presented in this dissertation is organized in manuscript format. Following this introduction and the literature review presented in Chapter 2, five studies are presented in Chapters 3 through 7. These research chapters are organized following the N cycle, beginning with the input of N into soil via N_2 fixing field pea and concluding by examining the influence of the two different crop species on the potential for N loss from soils via denitrification.

The goal of the research presented in Chapter 3 was to trace symbiotically fixed N_2 from field pea roots into the soil. A labeling system was designed to provide a continuous supply of $^{15}N_2$ to the roots and nodules of field pea grown under controlled conditions in a greenhouse. The specific objectives of the study were to determine N fixation throughout the lifecycle of field pea, as well as to determine the partitioning of fixed ^{15}N and N rhizodeposition in the soil at various developmental stages. Chapter 4 presents research with a similar objective of determining N rhizodeposition across field pea development, but employed an alternative ^{15}N labeling method. The cotton-wick method was used to ^{15}N-label field pea continuously to determine total N rhizodeposition. Although the release of symbiotically fixed N cannot be directly determined using cotton-wick ^{15}N labeling, the method is a technically simpler alternative to $^{15}N_2$ labeling and also may be used to estimate N rhizodeposition in non-legume plants.

Chapter 5 presents results from a study comparing N rhizodeposition of mature field pea and canola using the cotton-wick ^{15}N labeling method. The objective of this study was to quantify the contribution of belowground N (i.e., N in roots and rhizodeposits) to the total amount of N input into the soil from crop residues of these dissimilar crop species following seed harvest. The net contribution of root-derived N to soil inorganic N pools—either through mineralization of organic rhizodeposits or direct exudation of NH_4^+ and NO_3^-—at the end of the growing season was also determined. Chapter 6 furthers the work of the previous chapter by examining the influence of previous crops of field pea and canola on the growth of a subsequent wheat crop. The goal of this study was to differentiate between the supply of N from belowground versus aboveground residues of field pea and canola to wheat. In this study, a

cross-^{15}N-labeling approach was used to determine whether the aboveground and belowground residue-N supply to wheat differed within and among previous crop species. In Chapter 7, I investigated whether potential denitrification and the abundance of denitrifying genes differs in soils grown with field pea versus canola. In particular, I tested whether there was correlation between inputs of root-derived N and root biomass on potential denitrification and denitrifying gene abundance. Finally, Chapter 8 synthesizes the major findings of the research studies and suggests future work.

2. LITERATURE REVIEW

2.1. Prairie Cropping Systems

The cereal-fallow cropping systems that dominated agriculture on the Canadian prairies throughout most of the 20th century have been replaced by more diverse and intensive cropping systems. This shift was facilitated by the widespread adoption of no-tillage practices in the 1990s. By retaining crop residues on the soil surface, no-till management abated soil erosion and conserved soil moisture. As a result, the practice of summer fallowing became less necessary and the frequency of its practice has declined substantially in the last few decades—although to varying degrees depending on soil zone (Zentner et al., 2002). The improved soil moisture retention under no-till allowed producers to grow crops in the time and space previously dedicated to summer fallow, resulting in increased cropping intensity in this region (Grant et al., 2002). Moreover, the increase in soil moisture afforded by no-till increased the diversity of crops suited for the climate (Johnston et al., 2002), allowing producers to move away from cereal monocultures, which often are associated with persistence of crop disease (Lupwayi and Kennedy, 2007). While wheat remains the dominant field crop on the Prairies, this led the way for the introduction of pulse and oilseed crops into crop rotations with cereals.

Oilseeds well suited to the cool climate of the Canadian prairies include the *Brassica* species, canola and mustard, and flax (Gan et al., 2004; Johnston et al., 2002). Canola is the dominant oilseed crop grown in this region and throughout the country (Johnston et al., 2002). Moreover, second to wheat, canola is the most widely sown crop in Saskatchewan (Statistics Canada, 2011). Since improvement of oil quality and reduction of glucosinolates through breeding programs in the 1970s (May et al., 2010), the area sown to canola has grown steadily from 2 million to nearly 8 million ha across Canada, with over half of this area sown in Saskatchewan (Statistics Canada, 2011; May et al., 2010). Canola has a deep tap rooted system, allowing it to exploit water and nutrients deep into the profile (Gan et al., 2009a; Johnston et al., 2002), but also has a lower water use efficiency than wheat (Gan et al., 2009a), and has a relatively high N fertilizer requirement (Grant and Bailey, 1993). The influence of a preceding

crop of canola on subsequent crop yields have been mixed; with succeeding crop yields increasing (Brandt and Zentner, 1995; Soon and Clayton, 2002) or decreasing (Grant et al., 2009; Koide and Peoples, 2012; McGonigle et al., 2011) relative to other preceding crop species. Reduced yield of crops following canola may be attributed to reduced colonization of arbuscular mycorrhizal fungi (AMF)—canola does not associate with AMF, thus host availability is disrupted during the canola phase of the rotation (McGonigle et al., 2011).

Pulse crops are legumes grown for edible seed and are a relatively recent addition to crop rotations on the Canadian prairies. The pulse crops predominantly grown in this region include field pea, lentil, chickpea, faba bean, and dry bean, whereas soybean dominates production in eastern Canada. Field pea is the most widely sown pulse crop across the Canadian prairies and Canada is the world's largest producer and exporter of pea (Roy et al., 2010), with 68% being produced in Saskatchewan (Statistics Canada, 2011). Although occupying less area than wheat and canola, pulse crops have a strong foothold in Saskatchewan's agricultural industry as they are expected to play an important role in meeting current and future global protein demands (CBC, 2012). Indeed, pulse crops are highly nutritious and relatively inexpensive, contributing to their importance as an agricultural commodity (Roy et al., 2010). They are particularly valuable in crop rotations due to their ability to fix N. Improved cereal grain yields following pulse crops compared to cereal monoculture are well documented (Bremer et al., 2011; Gan et al., 2003; Krupinsky et al., 2006; Miller and Holmes, 2005; Miller et al., 2003b; Tanaka et al., 2007), with the yield increases attributed to both N and non-N factors (Stevenson and van Kessel, 1996a; Stevenson and van Kessel, 1996b).

The benefits associated with the diversification and intensification of cropping systems include increased yield potential (Grant et al., 2002), farm profitability (Zentner et al., 2002), improved soil nutrient retention and cycling (Gardner and Drinkwater, 2009; Liebig et al., 2006), and decreased carbon footprint (Gan et al., 2011). These agronomic and environmental benefits are attributed to a number of factors. For example, alternating crops reduces weed competition (Cardina et al., 2002; Seymour et al., 2012; Stevenson and van Kessel, 1996b) and breaks disease cycles (Krupinsky et al., 2002; Nayyar et al., 2009). Water use efficiency may be improved through strategic sequencing of crops; for example, by alternating pulse crops with oilseeds or wheat (Gan et al., 2009a). Increased soil organic matter and improved soil structure can occur due to a higher return of crop residues if production is increased (Grant et al., 2002). Indeed,

replacing summer fallow with crops and converting to no-till has resulted in an increase of soil organic C (SOC) on the prairies (VandenBygaart et al., 2003), but it is also true that the influence of continuous cropping sequences on changes in SOC can vary depending on the crops grown (Bremer et al., 2011; Lemke et al., 2007; VandenBygaart et al., 2003). Finally, benefits from crop diversity and intensification have been attributed to the fact that crops exert species-specific influences on soil nutrient cycles due to differences in nutrient requirements and utilization (Robertson and Vitousek, 2009), as well as differences in the quantity and quality of residues that remain following crop harvest (Grant et al., 2002). Therefore, the crops chosen and their sequence and frequency within crop rotations can influence nutrient cycles in intensified and diversified cropping systems.

2.2. Nitrogen Sources and Cycling in Agroecosystems

The vast majority of N in the biosphere derives from the atmosphere as a result of biological N_2 fixation (BNF). A group of microorganisms, collectively known as diazotrophs, are capable of converting dinitrogen gas (N_2) to ammonia (NH_3), which plants use to build amino acids and other N-associated compounds required to support growth. These free living, symbiotic, or plant-associative microorganisms use nitrogenase to catalyze the energy intensive reaction. In leguminous plants, BNF occurs in nodules that are formed on the roots following infection by *Rhizobium* bacteria. Rhizobia supplies N to the plant in exchange for C acquired from photosynthesis. This legume-rhizobium symbiosis provides the most important contribution of BNF in agricultural systems (Russelle, 2008), and until the mid-twentieth century, crop production was almost solely reliant on biological sources of N (Crews and Peoples, 2005).

The Haber-Bosch process is the industrial production of N fertilizer and is marked as the most important invention of the 20th century (Smil, 2001). The process uses high pressure and temperatures to overcome the lack of a suitable catalyst capable of cleaving the strong triple bond at room temperature (Gordon et al., 2001), which nitrogenase accomplishes naturally. The industrial production of N fertilizer requires 1% of the world's annual energy supply to produce the hydrogen and high pressure and temperatures necessary, making it a very costly process (Smith, 2002). The shift from biological to industrial sources of N resulted in massive yield increases, as inorganic N could be added directly to soils and availability of N to plants did not rely on the synchronization of N mineralized from organic sources. Indeed, N fertilizer was

pivotal in realizing the yield potential of the cereal hybrids bred during the Green Revolution. It has been suggested that 40% of the human population owes their existence to N fertilizer produced by the Haber-Bosch process (Smil, 2002). Certainly, the use of N fertilizer to increase crop production has had a profound influence on humanity—in turn, however, no other human activity has caused more significant change to the global N cycle than agriculture (Smil, 1999). Since the 1960s, human activity has more than doubled the rate of newly fixed N into the terrestrial N cycle, primarily due to N fertilizer (Vitousek et al., 1997).

Crops access only a portion—between 40 and 60%—of added inorganic N fertilizer to agroecosystems in the year of application (Janzen et al., 2003). Furthermore, plants generally access a higher amount of N mineralized from soil organic matter (>60%) than from inorganic fertilizer, even at high application rates (Gardner and Drinkwater, 2009). In an assessment of Canadian agricultural census data from a single year, Janzen et al. (2003) estimated that the amount of N inputs were twice that of N contained in harvested products. Rough estimates of N lost to the atmosphere and groundwater rival that of harvested N; therefore, only a small amount of added N may accumulate and cycle internally in the soil (Janzen et al., 2003). Within the season of application, 38% of added fertilizer N was not accounted for in plant and soil in a meta-analysis of a global data set from ^{15}N labeling studies in temperate agroecosystems, indicating a loss to the environment (Gardner and Drinkwater, 2009). Some N loss is inevitable (Janzen et al., 2003), but must be minimized to reduce the economic costs of N fertilizer use and to mitigate environmental harm.

Nitrogen leaves agroecosystems through leaching of NO_3^- to groundwater and as gaseous products during nitrification, denitrification, and ammonia volatilization. Reducing N fertilization to crop rotations with increased diversity can reduce the risk for groundwater contamination (Malhi et al., 2009). Moreover, the potential for nitrate leaching below the root zone is reduced in continuously cropped compared to fallow soils (Grant and Lafond, 1994). Nitrous oxide emissions increase with increasing rates of N fertilizer and, in the Canadian prairies, tend to be greater under conventional compared to no-till systems (Helgason et al., 2005; Malhi and Lemke, 2007; Malhi et al., 2006).

Nitrogen mineralized from crop residues and soil organic matter is made available for crop uptake, but is also susceptible to loss via the same pathways as N from fertilizer (Janzen et al., 2003). Environmental factors such as temperature and moisture influence N mineralization by

affecting soil microbial activity (Lupwayi and Kennedy, 2007). Nitrogen mineralization from soil and crop residues is negatively correlated to the C to N ratio (Booth et al., 2005; Heal et al., 1997) and positively correlated to N content (Lupwayi and Kennedy, 2007), although other residue quality factors (e.g., lignin content) also affect N mineralization (Heal et al., 1997). As a result, N mineralization rates (Lupwayi et al., 2006; Soon and Arshad, 2002) and the supply of N to subsequent crops (Soon and Arshad, 2004) varies among residues of different crop species. In addition, the effects that crop residues have on leaching (Kuo and Sainju, 1998; Thomsen and Christensen, 1996; Thomsen and Christensen, 1998) and N_2O emissions (Huang et al., 2004; Toma and Hatano, 2007) varies depending on the amount and quality of residues. Whereas N_2O emissions can be enhanced with the addition of crop residue relative to bare soils (Baggs et al., 2003), emissions from soils amended with crop residues may be less than from N-fertilized crops (Delgado et al., 2010). Moreover, the loss of N_2O from soils planted with legumes—which return residues of higher N concentration than non-legumes to the soil—generally are less than N-fertilized systems (Jensen et al., 2012). Nitrogen retention is more likely in systems that add N in conjunction with C (e.g., as crop residues) than when N is added alone as fertilizer (Gardner and Drinkwater, 2009). Therefore, management strategies that work to re-couple C and N cycles are proposed to mitigate N losses to the environment (Gardner and Drinkwater, 2009).

Legumes grown in agricultural systems represent an alternative to complete reliance on fertilizer to meet the N requirements of crop production. Moreover, because legumes utilize inorganic N when supplies are high and rely on BNF when N is limited, legumes can play a role in moderating inorganic N in soils (Russelle, 2008). The amount of N_2 fixed by legumes varies considerably among crop species (Peoples et al., 2009). For example, estimates of N fixation based on global averages of pulse crops grown in the Canadian prairies amounted to 58, 86, 51, 107, and 23 kg N ha^{-1} for chickpea, field pea, lentil, faba bean, and common bean, respectively (Gan et al., 2011; Herridge et al., 2008). The amount of fixed N is dependent on biomass yields and the plant's reliance on N fixation, which also varies among crop species and cultivars of a single species (Gan et al., 2010a; Soon and Lupwayi, 2008). In a comprehensive review of field studies in the Northern Great Plains, Walley et al. (2007) determined the median percentage of N derived from the atmosphere (%Ndfa) in field pea (55%), lentil (60%), kabuli and desi chick pea (<45 and 55%, respectively), faba bean (88%), and common bean (<45%). In field pea, N fixation was as low as 0 and as high as ~87%. The range in %Ndfa may be attributed partially to

Rhizobia strain and legume genotype interactions (Soon and Lupwayi, 2008). In addition, soil factors, including inorganic N availability, phosphorus (P) and micronutrient availability, soil pH, salinity and sodicity, and climate factors such as precipitation and temperature can have a significant influence on N_2 fixation (Peoples et al., 2009; Russelle, 2008).

A high proportion of plant N is removed from the field when pulse crop seeds are harvested (Peoples et al., 2009). As a result, the return of N in pulse crop residues to soil can be low (Lupwayi and Kennedy, 2007), and may not contribute to a net input of fixed N to the soil (Walley et al., 2007). Indeed, both positive and negative N budgets of pulse crops have been reported (Bremer et al., 2011; Gan et al., 2010a; Hauggaard-Nielsen et al., 2009; Haynes et al., 1993; Soon and Lupwayi, 2008; van Kessel, 1994). Even when there is a net depletion of N following pulse crops, positive N benefits to the yield and grain protein content of subsequent crops may still occur (Soon and Lupwayi, 2008). By relying on N fixation, pulse crops spare soil inorganic N for subsequent crops relative to non-legumes (Herridge et al., 1995; Jensen, 1994a). Moreover, mineralization of pulse crop residues of relatively low C to N ratios relative to cereal and oilseed crops also contributes to higher soil inorganic N levels observed following pulse crops (Lupwayi and Soon, 2009; Miller et al., 2003a; Soon and Arshad, 2002). The supply of N from pulse crop residues to subsequent crops is often higher than from cereal residues (Jensen, 1996a), although this is not always the case (Bremer and van Kessel, 1992a). In the short-term, the supply of residue-N to a succeeding crop may be relatively low (i.e., < 10%) (Bremer and van Kessel, 1992a; Jensen, 1994a; Jensen, 1996a), but helps to maintain soil organic matter and fertility over the long term (Lupwayi and Kennedy, 2007). In studies that examine the contribution of pulse crop residue N to subsequent crops, the belowground inputs of N are rarely (Lam et al., 2012; Mayer et al., 2003a; Russell and Fillery, 1996a; Soon and Arshad, 2004) if ever included (Lupwayi and Kennedy, 2007; Peoples et al., 2009). Therefore, the input of N_2 fixation to the overall N budget and the supply of N from pulse crop residues to subsequent crops are likely underestimated.

Nitrogen contained in crop roots is difficult to assess because standard methods of root excavation and root washing result in poor root recovery (Subedi et al., 2006; Wichern et al., 2008). Moreover, these methods do not assess N released through rhizodeposition, which may comprise a significant recycling of N from plant to soil. Rhizodeposition is defined as the release of inorganic and organic compounds from roots during plant growth (Nguyen, 2003; Wichern et

al., 2008). These compounds may be water insoluble such as sloughed root cells, mucilage, and cell walls or may be water soluble exudates such as amino acids, organic acids, sugars, hormones and vitamins (Uren, 2007; Wichern et al., 2008). The amount and type of compounds released varies among crop species, according to the plant physiological status, spatially along the root, and temporally as plants develop (Dennis et al., 2010; Jones et al., 2009; Nguyen, 2003; Van der Krift et al., 2001). Based on an updated review of rhizodeposition, Jones et al. (2009), estimated that rhizodeposition comprises approximately 11% of net photosynthetic C. Studies that examine N rhizodeposition are less predominant (Jones et al., 2009; Wichern et al., 2008), but indicate that N rhizodeposition comprises between 2.7 and 71.1% of total plant N for legumes (Wichern et al., 2008) and between 4.3 and 56.0% for cereals (Wichern et al., 2008). Nitrogen released to soil from plant roots occurs as NH_4^+, NO_3^-, amino acids, cell lysates, sloughed roots, and other root-derived debris (Brimecombe et al., 2007).

Rhizodeposition represents a significant energy source that drives many of the microbial processes that occur in the rhizosphere (Merbach et al., 1999). Rhizosphere soils harbour unique microbial communities, which are influenced both by the indigenous microbial community and plant species (Hawkes et al., 2007). Indeed, many studies have demonstrated a high specificity of microorganisms to plant species and even to cultivars of a single plant species (Berg and Smalla, 2009). Rhizosphere microbial community structure is strongly determined by rhizodeposition (Paterson et al., 2007), with rhizodeposition pools other than root exudates (e.g., cell lysates) playing a more significant role (Dennis et al., 2010). Studies examining the relationship between rhizodeposition and microbial community structure and function (e.g., denitrification) have focused on C (Henry et al., 2008; Paterson et al., 2007). Differences in N rhizodeposition determined in pea, white lupin, and faba bean (Mayer et al., 2003b), were hypothesized to explain the different structure of denitrifying bacteria transcripts in the rhizospheres of these crops in a related study (Sharma et al., 2005). However, these latter authors did not determine N rhizodeposition directly. Therefore, the effect of N rhizodeposition on soil microbial communities, and in particular, on specific functional groups involved in the N cycle is not known.

2.3. ^{15}N Labeling Techniques to Determine N Rhizodeposition[1]

Methods that directly supply plants with ^{15}N have been developed primarily for investigations of N rhizodeposition, with the ^{15}N label being used to differentiate the small input of N from roots against the large background of soil N (Schmidtke, 2005a). Interest in developing methods to determine N rhizodeposition has increased in the last decade as scientists seek to understand the contribution of belowground N to total N budgets, particularly in legume-based crop rotation and intercropping systems (Fustec et al., 2010), and the fate of rhizodeposits from legumes and cereals in the various soil N pools (De Graaff et al., 2007; Janzen, 1990; Jensen, 1996c; Mayer et al., 2004; Schenck zu Schweinsberg-Mickan et al., 2010; Wichern et al., 2007b). Despite our limited understanding of the ecological significance of N rhizodeposition (Wichern et al., 2008), few ^{15}N studies have investigated the chemical composition of the organic N compounds released to the soil from plant roots (Hertenberger and Wanek, 2004; Merbach et al., 1999). While there are clear conceptual definitions of rhizodeposition, elucidating differences in processes through experimentation remains difficult (Jones et al., 2009). Due to methodological challenges even relatively recent studies on N rhizodeposition have focused on improvement and comparison of ^{15}N stable isotope techniques (Hertenberger and Wanek, 2004; Khan et al., 2002a; Mahieu et al., 2009a; Mahieu et al., 2007; Schmidtke, 2005b; Wichern et al., 2011; Yasmin et al., 2006). Indeed, the methods developed to determine C and, particularly, N rhizodeposition are fraught with uncertainty, primarily due to difficulties in achieving label uniformity within the plant and thus difficulties satisfying the assumptions of the calculations involved (Rasmussen, 2011).

Most ^{15}N isotope methods take the approach of supplying the tracer directly to the plant without labeling the soil and include: shoot labeling (Mayer et al., 2003b; Russell and Fillery, 1996b; Wichern et al., 2007a; Wichern et al., 2007b); leaf immersion (De Graaff et al., 2007; Khan et al., 2002b); and atmospheric labeling using NH_3 (Janzen and Bruinsma, 1989; Schulze and Merbach, 2008) or N_2 (Mohr et al., 1998; Russelle et al., 1994). Only split-root labeling introduces the ^{15}N label to the plant through the soil (Jensen, 1996c; Mahieu et al., 2009b). Of

[1]Section 2.3 is a condensed version of the section entitled "^{15}N Isotope Techniques" that was previously published in: Leinweber, P., J. Kruse, C. Baum, M. Arcand, J.D. Knight, R. Farrell, K.-U. Eckhardt, K. Kiersch, and G. Jandl. 2013. Advances in understanding organic nitrogen chemistry in soils using state-of-the-art analytical techniques, In: D. L. Sparks, editor, Adv. Agron. Academic Press. p. 83-151. The research and writing of this section was done by me and was edited by R. Farrell and J.D. Knight with minor edits by P. Leinweber.

these methods, the atmospheric labeling of N_2 and NH_3, and the split-root technique assimilate ^{15}N into the plant via natural mechanisms.

Application of ^{15}N-enriched solutions to the aerial parts of the plant is the most common method used to label roots and ultimately the soil through rhizodeposition. These methods are technically simple to implement for a variety of crop species and do not require complicated or expensive equipment. Leaf and petiole labeling involves cutting the leaf and immersing the cut leaf or petiole in a vial containing the ^{15}N labeled solution. Alternatively, the ^{15}N solution can be supplied to the plant via a cotton wick, threaded into the stem, and immersed in ^{15}N-enriched solution contained in a vial, and is often referred to as the stem- or cotton-wick technique. The transpiration stream drives the ^{15}N uptake using stem-wick feeding, whereas active and passive transport mechanisms are involved in transfer of ^{15}N using leaf immersion techniques (Russell and Fillery, 1996b). Solution uptake efficiency generally follows the order: cut leaf feeding > petiole feeding > stem-wick feeding (McNeill et al., 1997; Yasmin et al., 2006). Whereas Khan et al. (2002b) suggested that leaf and petiole feeding may be suitable for short-duration ^{15}N labeling in the field, the stem-wick feeding apparatus is more robust and is suitable for long-duration labeling in the field (Mahieu et al., 2007; Wichern et al., 2007a)—though labeling frequency may be limited if field sites are relatively remote.

Labeling method, frequency of ^{15}N labeling, and type and concentration of labeling solution used in leaf- and shoot-labeling experiments influences ^{15}N enrichment of roots and soil, thus influencing calculations of N rhizodeposition. Leaf- and shoot-labeling studies most often use highly enriched (99 atom% ^{15}N) urea as a carrier for the ^{15}N label (Khan et al., 2002b; Russell and Fillery, 1996b) because it is non-polar, has a high N to mass ratio, and may be readily metabolized by urease within the plant (McNeill, 2001). However, solutions of NH_4Cl (Gotz and Herzog, 2000) and KNO_3 (De Graaff et al., 2007) also have been used. Mahieu et al. (2009a) observed a linear relationship between the atom% ^{15}N of roots and soil when pea was supplied with urea at concentrations of 0.2–0.6%. However, this relationship did not hold, and ^{15}N soil enrichment was higher and more variable, when the urea concentration was 0.8%. The authors suggested that since plant urease may not be responsive to high exogenous additions of urea, temporary buildup of the urea in plant organs can occur. This will affect the composition and distribution of the ^{15}N within the plant if the urea is not metabolized by urease, and will have consequences for the assessment of N rhizodeposition (Mahieu et al., 2009a). In addition, high

concentrations of urea can cause necrosis, though the concentration threshold [0.5 % to 2.0 % (w/v)] depends on plant species (Khan et al., 2002b; Mahieu et al., 2009a; Russell and Fillery, 1996b).

Labeling frequency can vary from a single application of ^{15}N (Khan et al., 2002b; Yasmin et al., 2006) to fortnightly or weekly ^{15}N pulses (Mahieu et al., 2007; Mayer et al., 2003b; Russell and Fillery, 1996b), or ^{15}N pulses at specific growth stages (Mahieu et al., 2007; Wichern et al., 2007a); to continuous application (Mahieu et al., 2009a). Mahieu et al. (2007) reported that fortnightly pulses of urea resulted in higher soil ^{15}N enrichment than pulses applied at specific growth stages. In a subsequent study, labeling frequency did not influence root ^{15}N enrichment; though, more frequent labeling (continuous *vs.* fortnightly pulses) increased soil ^{15}N enrichment in field pea through rhizodeposition—indicating that continuous labeling resulted in higher and more reliable estimates of N rhizodeposition (Mahieu et al., 2009a). Regardless of ^{15}N-labeling frequency, the root distribution ratios (distribution of ^{15}N in roots to distribution of total N in roots) were always <1 (Mahieu et al., 2009a), indicating that labeling frequency does not always improve the distribution of ^{15}N within the whole plant. Therefore, more frequent application of ^{15}N labeled urea can increase ^{15}N enrichment of the soil—improving quantification of N rhizodeposition—even though distribution of the ^{15}N may still favor aboveground components. In some studies, application rates of ^{15}N have been matched with plant N demand in an attempt to improve label uniformity (Mahieu et al., 2007; Mayer et al., 2003b; Russell and Fillery, 1996b). Despite best efforts to improve ^{15}N homogeneity by applying a continuous supply of ^{15}N in concert with whole-plant demand, variations in the N sink strength within the plant that occur throughout the growth cycle can potentially result in heterogeneous distribution of ^{15}N (Mahieu et al., 2009a).

The split-root technique involves splitting the root system of a single plant or plants into two compartments: one that is supplied with ^{15}N fertilizer and the other, which is sampled and analyzed for ^{15}N enrichment in the roots and rhizodepositional transfer to the soil (Sawatsky and Soper, 1991). This technique enables continuous labeling through a natural uptake mechanism and provides relatively homogeneous root enrichment (Wichern et al., 2008). Root ^{15}N enrichment is greater when labeled with split-root techniques compared to shoot-labeling techniques (Schmidtke, 2005a), but ^{15}N recoveries tend to be lower (Mahieu et al., 2007). Schmidtke (2005b) suggested that N rhizodeposition as a proportion of total plant N is

underestimated using split-root techniques because only half of the root system is considered; however, the results of Mahieu et al. (2007) indicate that the converse may be true. They determined that the ratio of N derived from rhizodeposition (NdfR) to total plant N was 10% higher in split-root compared to stem-wicked pea plants. However, because the relationship between root and soil ^{15}N enrichment differed between methods, they could not resolve which of the two labeling techniques produced the most 'correct' estimate of rhizodeposition.

Atmospheric labeling of $^{15}N_2$ can be used to assess the proportion of symbiotically or non-symbiotically fixed N to soil by legumes (Mohr et al., 1998; Russell and Fillery, 1996b) and non-legumes (Bremer et al., 1995), respectively. Plants may be labeled with ^{15}N by exposing the root system to an atmosphere enriched in $^{15}N_2$, either continuously or as one or a series of short-duration labeling periods to determine symbiotic or associative N_2 fixation. A greater proportion of fixed ^{15}N will be recovered in soil N pools under continuous labeling and this technique yields results that are more representative of the N fixed during the lifecycle of the plant (Mohr et al., 1998) compared to short duration pulses (Russelle et al., 1994). However, the equipment required to simultaneously regulate $^{15}N_2$, O_2, and CO_2 concentrations is both expensive and technically complicated, which limits widespread adoption of this method and restricts it to controlled environment experiments. Despite the high cost and technical difficulty, direct labeling of legumes by root and nodule exposure to $^{15}N_2$ is the most accurate measure of N_2 fixation compared to other methods (Warembourg, 1993), and therefore provides a true representation of the contribution of fixed N to soil through N rhizodeposition of legumes.

3. DETECTING BIOLOGICALLY FIXED-N IN THE RHIZOSPHERE OF FIELD PEA UNDER CONTROLLED CONDITIONS

3.1. Preface

Pulse crops are often touted for improving the soil N supply to subsequent crops as a result of their ability to fix N_2 in symbiotic association with *Rhizobium* bacteria. A large proportion of total pulse crop-N is removed when seed is harvested, thus reducing the amount of fixed N entering the soil system. As a result, the only inputs of N to soil from legumes grown for seed are those derived from N remaining in roots, rhizodeposits, and straw residues. Despite this, N balance studies often do not account for root and rhizodeposit N, thus underestimating the fixed-N contribution of pulse crops. Moreover, analysis of the N content in roots that are physically recovered at crop maturity does not account for the N released to soil through rhizodeposition or decomposition of roots that die prior to crop harvest. Nitrogen-15 isotope techniques have been developed to trace the release of N from plant roots, thus improving the accounting of total plant N. More specifically, direct assessment of N_2 fixation and the release of fixed-N to soil from legume roots can be made if nodules are exposed to a soil atmosphere that is enriched in $^{15}N_2$ throughout plant growth. However, studies using atmospheric $^{15}N_2$ labeling of pea have been concerned with evaluating factors that influence N_2 fixation specifically, rather than the input and fate of fixed-N in soil. The goal of this study was to determine the contribution of fixed N to soil from field pea at different stages of plant growth using a closed continuous $^{15}N_2$ atmospheric labeling approach. Due to the complexity of the apparatus used to control atmospheric conditions, the experiment was conducted in a greenhouse.

3.2. Abstract

Continuous exposure of nodulated legume roots to $^{15}N_2$ provides the most direct measure of symbiotic N_2 fixation. Moreover, the use of $^{15}N_2$ labeling allows for the tracing of fixed N to soil through the process of rhizodeposition. Under controlled conditions in a greenhouse, field pea was grown and the roots exposed to a soil atmosphere enriched in $^{15}N_2$ continuously from eight

leaves unfolded through harvest. Plants were destructively sampled at the vegetative stage, flowering, pod filling, and maturity. Leaks in the air circulation system together with relatively low nodulation resulted in low measures of N_2 fixation by the field pea. Nevertheless, N_2 fixation was active, as evidenced by a high ^{15}N enrichment in the nodules (1.6343 atom% ^{15}N excess averaged over all growth stages). Despite low levels of N_2 fixation—N derived from the atmosphere represented only 2.7, 2.0, 5.7, and 7.8% of total plant N at the vegetative stage, flowering, pod filling, and maturity, respectively—there was a significant positive relationship between atom% ^{15}N excess in the roots and atom% ^{15}N excess in the rhizosphere soil, indicating that fixed N was released to soil. The results of this study highlight the fact that the input of symbiotically fixed N in pulse cropping systems is not always guaranteed—low N_2 fixation rates were, at least partially, attributable to relatively high soil nitrate levels. Indeed, while there were small amounts of fixed N released into the soil over the course of crop growth, the reliance on soil N reserves rather than N_2 fixation resulted in the net export of fixed N from the system once the seed was harvested.

3.3. Introduction

The positive influence of legumes on soil quality and the overall productivity of agricultural systems has been known for over two millennia (van Kessel and Hartley, 2000). Despite this, only in the last two decades have legumes become an important component of the agricultural industry in the Canadian Prairies. In particular, production of pulse crops (i.e., legumes grown for seed consumption) has increased across the Canadian prairies and together with the adoption of other practices, such as no-till, have improved the economic and environmental sustainability of agriculture in this region (Cutforth et al., 2007). Farmers have reaped the economic benefits of increased cropping intensification facilitated by no-till and have replaced fallow periods with pulse crops (Lupwayi and Kennedy, 2007). In addition, the combined benefits of N and water use efficiency make pulse crops well suited for production in these semi-arid environments (Miller et al., 2002). Diversification reduces economic risk, as pulse crop prices are somewhat detached from cereals, and offers more crop choices in the face of climate change (Cutforth et al., 2007). During the last two decades, field pea (*Pisum sativum*) has been the dominant pulse crop (Lupwayi and Kennedy, 2007).

Growing pulse crops can save on fertilizer costs by relying on symbiotic N_2 fixation to satisfy a portion of the N requirement and can confer both N and non-N benefits to succeeding crops (Stevenson and van Kessel, 1996a). In particular, pulse crops may improve N availability to subsequent crops through the decomposition of N-rich crop residues or due to an N-sparing effect whereby soil N is conserved for the next crop (Herridge et al., 1995). Increasing the N input from pulse crops depends on whether the plant is obtaining most of its N from symbiotic N_2 fixation or from soil N—a net input of fixed N into the soil can only occur when the amount of fixed N contained in the remaining crop residues exceeds the amount of soil N removed in the harvested seed (van Kessel and Hartley, 2000). Indeed, a positive input of fixed N to soil following seed harvest of pulse crops is not guaranteed. An analysis of published results of N_2 fixation from a variety of pulse crops grown in the Northern Great Plains revealed that a positive contribution of fixed N is more likely to occur for high N-fixing crops such as faba bean (*Vicia faba*), field pea, and lentil (*Lens culinaris)*, while moderate N-fixing crops including desi and kabuli chickpea (*Cicer arietinum*) and common bean (*Phaseolus vulgaris*) are more likely to result in a N deficit (Walley et al., 2007). Based on the regression analysis of 79 reports of N_2 fixation and N increment in field pea, Walley et al. (2007) determined that a positive N input to soil would require that the plant acquire at least 47% of its N from fixation. Clearly, assessing N budgets for pulse crops and optimizing N management in these cropping systems relies on an accurate accounting of N in the crop residues that remain following seed harvest.

Biological N_2 fixation (BNF) can be assessed using stable isotope or non-isotope techniques. However, stable isotope techniques are the benchmark against which other techniques for determining N_2 fixation should be measured (Peoples et al., 2008). Isotope dilution approaches are most common and offer the advantages of providing a yield independent and time-integrated estimate of the percentage of N derived from the atmosphere (Chalk et al., 2010; Peoples et al., 2008). However, accurate determination of N concentration and biomass are required for determination of amounts of fixed N. Despite the acknowledgement that belowground biomass is an important component of total plant biomass for determining the N budgets of legumes (Chalk et al., 2010; Herridge et al., 2008; Peoples et al., 2008; Walley et al., 2007), logistical challenges in physical root recovery, as well as methodological challenges in quantifying N released through rhizodeposition, render assessments of the amounts of fixed N in belowground components difficult. In recent years, ^{15}N labeling studies have been developed and

have shown that legume roots can release large amounts of N—ranging from 4 to 71% of total plant N—to soil during crop growth through rhizodeposition (Fustec et al., 2010; Wichern et al., 2008). However, many of these methods are based on artificial assimilation of ^{15}N into the plant or disruption of the root system, and do not directly determine the release of fixed-N to the soil.

Atmospheric $^{15}N_2$ labeling is the most direct way to measure BNF and the contribution of fixed-N from roots to soil. However, the number of studies using $^{15}N_2$ to examine N_2 fixation in field pea are limited (Fischinger and Schulze, 2010; Oghoghorie and Pate, 1972; Sims et al., 1986; Voisin et al., 2003). The technical difficulty in establishing a controlled atmosphere in the rhizosphere, together with the complexity of the equipment required and the high cost of the $^{15}N_2$ labeled gas have limited its use (McNeill et al., 1994). Despite this, a few studies have used $^{15}N_2$ labeling to determine the amount of fixed-N released to soil from perennial forage legumes such as alfalfa (*Medicago sativa*) (Mohr et al., 1998; Russelle et al., 1994) and white clover (*Trifolium repens*) (McNeill et al., 1994), and the grain legume, common bean (Ruschel et al., 1979). Although reliable estimates of the input of fixed N to soil via rhizodeposition are vital to an accurate assessment of the N economy of legume-based cropping systems, to our knowledge no studies using $^{15}N_2$ labeling have been conducted to determine the amount of fixed-N released to the soil through rhizodeposition in field pea.

The objective of this study was to trace the release of fixed N to soil throughout the life cycle of field pea grown under controlled conditions using continuous $^{15}N_2$ labeling. The roots and nodules of field pea were exposed to $^{15}N_2$ in the soil atmosphere from eight leaves unfolded through harvest, with plants destructively sampled during the vegetative stage and at flowering, pod filling, and maturity. Abundance of ^{15}N in the aboveground plant components and roots was determined to evaluate the contribution of BNF to plant N and ^{15}N in soil was determined to evaluate the contribution of fixed N released to the soil during plant growth.

3.4. Materials and Methods

3.4.1. Soil preparation and planting

Soil, classified as an Orthic Dark Brown Chernozem, was collected from the Agriculture and Agri-food Canada research station at Scott, SK in the spring of 2009. The soils were from field plots that had been in the wheat phase of a pea-wheat rotation during the previous (2008) growing season. The soil was air-dried, sieved (4 mm) to remove any rocks, and mixed with

silica sand in a 1:1 ratio by weight to facilitate root recovery. The resulting soil-sand mix was classed as a loam with a pH of 5.6 (1:2 w/v soil:H_2O) and contained 1.3 g total N kg^{-1}, 77.3 mg NO_3^--N kg^{-1}, 103.1 mg P kg^{-1}, 734.3 mg K kg^{-1}, and 12.3 mg S kg^{-1}. Soils were packed to a bulk density of 1.3 Mg m^{-3} in pots (12 cm dia., 30 cm deep) constructed of polycarbonate pipe and were adjusted to 80% (w/w) water-holding capacity with deionized water. Pots were stored at 2°C and then placed on custom made weigh scales on a greenhouse bench prior to planting. Five pea seeds (cv. CDC Meadow), inoculated with *Rhizobium leguminosarum* (Nodulator® sterilized peat-based, Becker Underwood, Saskatoon, SK) at 1.5 times the recommended rate, were sown into each pot and thinned to one plant per pot following germination. In total, there were six replicate plants for each growth phase—yielding a total of 24 plants subjected to $^{15}N_2$ labeling. An additional three replicate plants per growth phase were grown without $^{15}N_2$ labeling in an adjacent greenhouse as natural abundance controls. Pot weight was monitored daily and the plants watered every one to two days to maintain the soil water content at 80 ± 3% field capacity.

At the 5-leaf stage, each pot was sealed with a cap that was fitted securely to the top of the pot and sealed with a gasket, leaving approximately 4 cm of headspace between the soil surface and the bottom of the cap. The shoot was fed through a hole (3 cm i.d.) in the cap such that there was no damage to the leaves or stem. The hole was then sealed using medical grade silicone (Silastic® MDX-4-4210, Dow Corning, Midland, Michigan), which was topped with a layer of a lanolin/paraffin wax mixture (1:2 w/w). Water was supplied using a syringe that could be attached to a luer lock on a two-way stop-cock that was fitted with tubing to the cap of the pot.

3.4.2. ^{15}N labeling system and gas sampling and analysis

Each of the 24 individual pots was connected to a central, closed gas circulating system consisting of components manufactured by Qubit Systems, Kingston, ON (Fig. 3.1) with an air flow rate of approximately 100 mL min^{-1}. The inlet port for each pot was located in the outside wall, *ca*. 8-cm above the bottom of the pot; the gas outlet port was located in the cap at the top of the pot (Fig. 3.2). A sampling port located halfway up the side of the pot was used for injection of the $^{15}N_2$ gas and to sample the soil atmosphere. The $^{15}N_2$ gas (99 atom% ^{15}N) was passed through a liquid N trap to remove any impurities (Warembourg, 1993) and stored in a Tedlar® gasbag prior to being injected into the pots. The first $^{15}N_2$ injection began approximately four weeks after emergence, corresponding to the plant having eight leaves unfolded. The initial

atmospheric labeling involved supplying each plant with 120 mL of pure $^{15}N_2$ (99 atom% ^{15}N) using a needle and syringe to inject the gas into the soil through the injection port on the sidewall of the pot. Oxygen was also supplied to ensure that the soil atmosphere consisted of approximately 80% N_2 and 20% O_2. During the first few days of $^{15}N_2$ labeling, it was found that the $^{15}N_2$ content of the atmosphere was rapidly depleted when the pump was running in continuous mode. Thereafter, the atmosphere in the system was circulated by running the pump for 15 min every 6 h. The roots and nodules of peas harvested at the vegetative stage (13 leaves unfolded), flowering, pod filling, and maturity were exposed to the $^{15}N_2$ for 10, 22, 42, and 57 d, respectively.

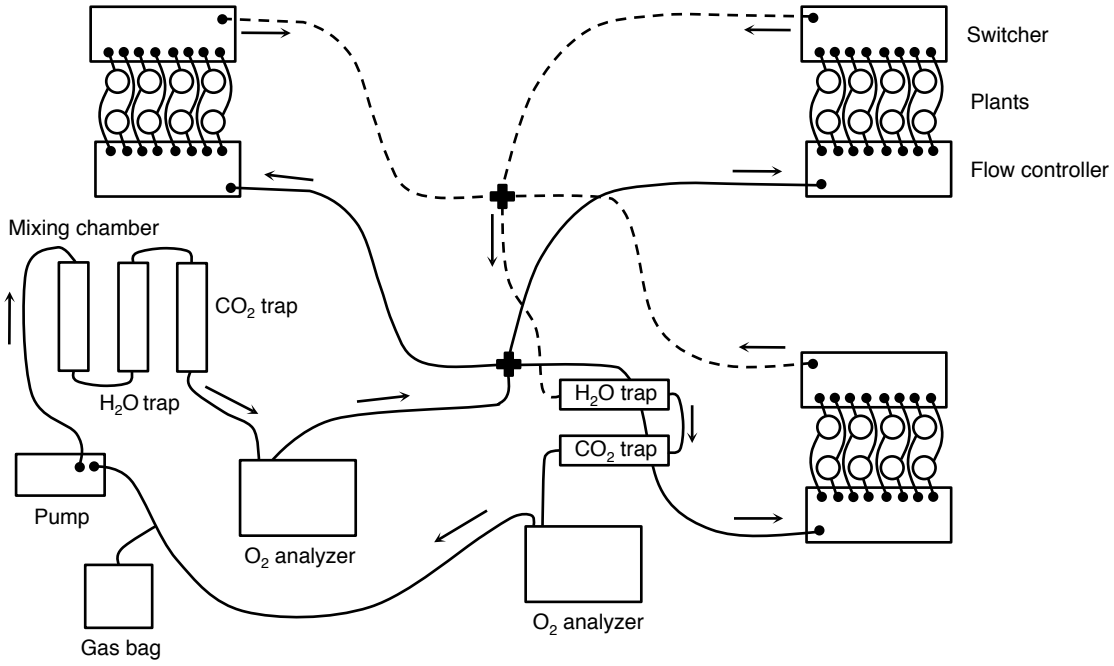

Fig. 3.1. Schematic of closed loop $^{15}N_2$ gas labeling system (not to scale). Arrows indicate direction of gas flow.

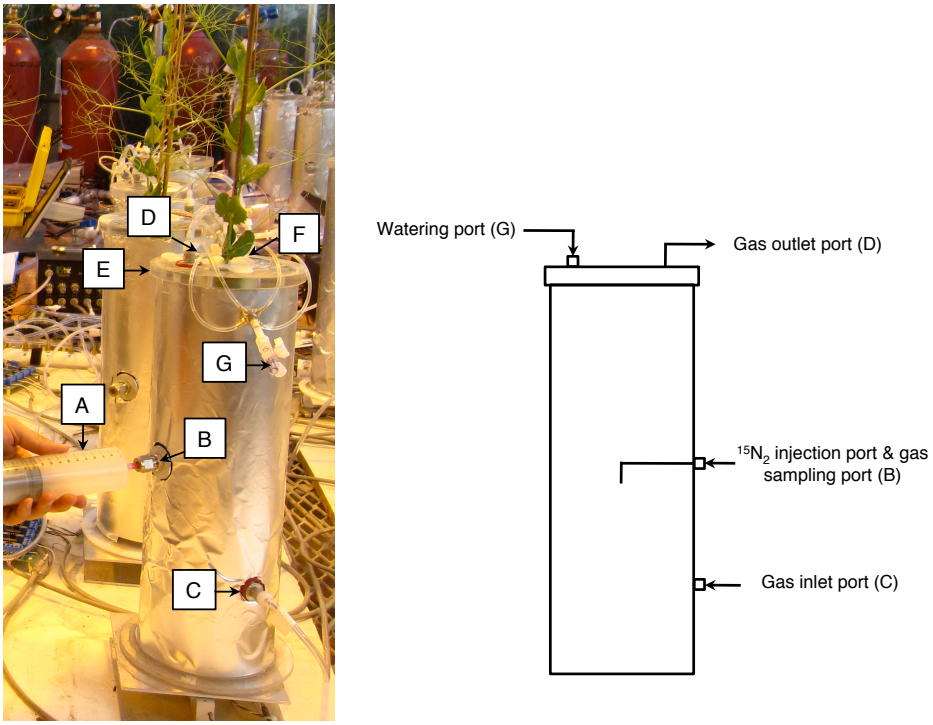

Fig. 3.2. Photograph and schematic of a pot containing a single pea plant that was connected to the $^{15}N_2$ gas-labeling system designed to continuously, and simultaneously, label 24 plants. A syringe (A) was used to manually inject $^{15}N_2$ into the injection port (B). The soil atmosphere was circulated among the 24 pots to replenish O_2, remove CO_2, and circulate $^{15}N_2$ via an inlet (C) and outlet (D) port. The plant stem was fed through a hole in an acrylic cap (E), which was sealed with medical grade silicone and paraffin/lanolin wax (F). Plants were watered manually via a two-way luer lock stopcock (G). Photograph published with permission from Elsevier.

A single 22 mL gas sample from each pot was collected daily and stored in a pre-evacuated 12 mL Exetainer® vial (Labco Ltd., UK). Following collection of the sample, fresh $^{15}N_2$ and O_2 were injected directly into each pot to replace the $^{15}N_2$ lost due to leaks in the system and to replenish the O_2 that was consumed by root respiration. An expansion bag was installed in-line with the system to regulate pressure changes occurring during gas injection and sampling. Excess CO_2 produced during root respiration was absorbed by soda lime contained in two columns connected to the gas circulating system; water vapour was absorbed by Drierite (WA Hammond Drierite Co., Xenio, OH) in an in-line column connected to the system. Real-time measurements of the O_2 concentration within the gas circulating system were made using two in-line O_2 meters (S108 Absolute O_2 Analyzer, Qubit Systems, Kingston, ON). The ^{15}N enrichment of the soil atmosphere was monitored by analyzing a subset of the gas samples (*ca*. every second day) for

atom% ^{15}N using a Costech ECS4010 elemental analyzer (Costech Analytical Technologies, Inc., Valencia, CA) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). A 1 mL subsample was collected from the Exetainer® vial for each gas sample, injected into a pre-evacuated 3.7 mL Exetainer® vial and filled with ultrapure Ar at atmospheric pressure. The gas samples were then analyzed for O_2 and CO_2 concentrations using gas chromatography (Varian CP-4900 Micro Gas Chromatograph, Varian Canada, Mississauga, ON).

3.4.3. Plant harvest and sample analysis

Plants were harvested during the vegetative stage at 33 days after sowing (DAS), then at flowering (45 DAS), pod filling (65 DAS), and maturity (86 DAS). At each harvest, the aboveground plant parts (leaves, stems, pods, grain) were separated, dried, and finely ground in a ball mill. Visible roots and root fragments were sampled from the soil using tweezers and a 2 mm sieve. Any soil directly adhering to the roots was considered rhizosphere soil and was collected by washing the roots with deionized water on a 1 mm sieve. The soil-water slurry was collected and the water evaporated in an oven at 70°C to recover the rhizosphere soil. The soil that remained following root sampling was considered bulk soil; all soil samples were dried and finely ground in a ball mill. Nodules were counted and removed from the washed roots, then dried and finely ground in a ball mill. All soil and plant materials were analyzed for N concentration (%) and atom% ^{15}N using the isotope ratio mass spectrometer coupled to the elemental analyzer.

3.4.4. Calculations

Nitrogen derived from the atmosphere (%Ndfa) in pea and N derived from rhizodeposition (%NdfR) was calculated for nodulated plants only. Nitrogen derived from the atmosphere was calculated as (Warembourg, 1993):

$$\%\text{Ndfa} = \frac{\text{atom\% } ^{15}\text{N excess plant}}{\text{atom\% } ^{15}\text{N excess soil atmosphere}} \quad [3.1]$$

Nitrogen derived from rhizodeposition was calculated as (Janzen and Bruinsma, 1989):

$$\%\text{NdfR} = \frac{\text{atom\% } ^{15}\text{N excess soil}}{\text{atom\% } ^{15}\text{N excess roots}} \quad [3.2]$$

Atom% ^{15}N excess values for the soil and plant components were determined by subtracting the total atom% ^{15}N in soil and plant components of pea grown under natural abundance conditions from the total atom% ^{15}N of plants that were exposed to a soil atmosphere enriched in $^{15}N_2$. Atom% ^{15}N excess values for the soil atmosphere were determined by subtracting the natural abundance (0.3663 atom% ^{15}N) of the atmosphere from the atom% ^{15}N values measured in the pots of plants exposed to $^{15}N_2$. The atom% ^{15}N excess root values included nodules. The %Ndfa values were calculated on a whole-plant basis. Nitrogen-15 enrichment data is presented for nodulated plants only, while biomass and N yields are presented for all plants, regardless of whether there was effective nodulation.

3.4.5. Statistics

The relationships between measures of field pea N_2 fixation (e.g., root and nodule ^{15}N enrichment and nodulation) and N rhizodeposition, as determined by rhizosphere soil ^{15}N enrichment, were evaluated using linear regression. Normality of residuals was tested using the Shapiro-Wilk statistic and homogeneity of variances was tested using Levene's test. To evaluate the effect of the labeling system on plant biomass and plant N at different growth stages, data were subjected to two-way analysis of variance (ANOVA) with labeling treatment (control *vs*. apparatus) and harvest stage as the main effects. In addition, total atom% ^{15}N data were subjected to two-way analysis of variance to determine whether ^{15}N enrichment of plant parts differed between control and $^{15}N_2$-exposed plants. All data were log transformed to meet the assumptions of the ANOVA. Means comparisons were made using Tukey's Honestly Significant Difference (HSD) test. All tests were declared significant at $P \leq 0.05$. Statistical analyses were performed using SPSS® Statistics version 20.0 for Mac (IBM Corp., 2011).

3.5. Results

3.5.1 Soil atmosphere ^{15}N enrichment

The ^{15}N enrichment of the soil atmosphere within pots connected to the closed loop system was maintained between 0.1747 ± 0.0146 and 4.8615 ± 0.3653 atom% ^{15}N excess per sampling period over the course of the experiment (mean ± s.d.; Fig. 3.3). Atom% ^{15}N declined sharply at the beginning of the experiment; then as the system stabilized the ^{15}N enrichment of the atmosphere remained relatively constant. That is, up till the harvest at pod filling (65 DAS), at

which point a leak was inadvertently created during the plant harvest. The mean ± s.d. ^{15}N enrichment in the soil atmosphere was 3.9317 ± 0.7842, 3.6112 ± 0.6185, 3.3931 ± 0.5896, and 3.0634 ± 1.0380 atom% ^{15}N excess over the course of exposure for plants harvested at the vegetative stage, at flowering, pod filling, and maturity, respectively.

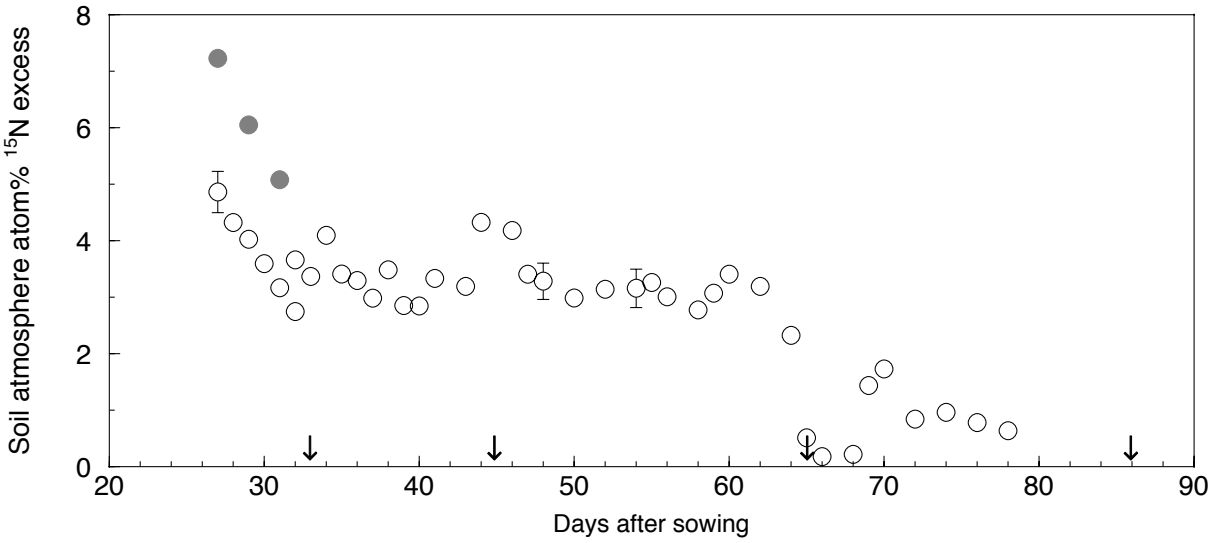

Fig. 3.3. Soil atmosphere atom% ^{15}N over the course of the labeling experiment. The arrows correspond to plant harvest dates: 13 leaves unfolded (33 DAS), flowering (45 DAS), pod filling (65 DAS), and maturity (86 DAS). Open circles represent means and error bars indicate standard deviations; the number of samples analyzed decreased over time as plants were harvested: n=9 until vegetative harvest; n=8, from vegetative to flowering; n=7 from flowering to pod filling; and n=3 from pod filling to maturity. Shaded circles represent data from a single pot that was identified as an outlier.

3.5.2. Plant growth and N uptake

Total plant biomass and N uptake increased throughout vegetative growth and flowering, then tapered off between pod filling and maturity (Fig. 3.4). Biomass and N uptake were consistently higher for plants grown within the $^{15}N_2$ labeling system compared to the control plants grown under ambient atmospheric conditions—there was no significant interaction between harvest stage and labeling treatment for biomass and N uptake. To avoid potential contamination from leaked $^{15}N_2$, the natural abundance control plants were grown in an adjacent greenhouse that was slightly shaded relative to that in which the ^{15}N-labeled plants were grown. Moreover, evapotranspirative loss from the control plants (determined from changes in pot weight) was up to 2-fold greater per day compared to $^{15}N_2$ labeled plants. This may explain the

difference in plant biomass and N uptake between the labeling treatments. Nodulation was low and variable over the course of the experiment—with the greatest number of nodules occurring at pod filling for plants grown in the $^{15}N_2$ labeling system (Fig. 3.5: max = 82 nodules plant^{-1}; median = 51 nodules plant^{-1}).

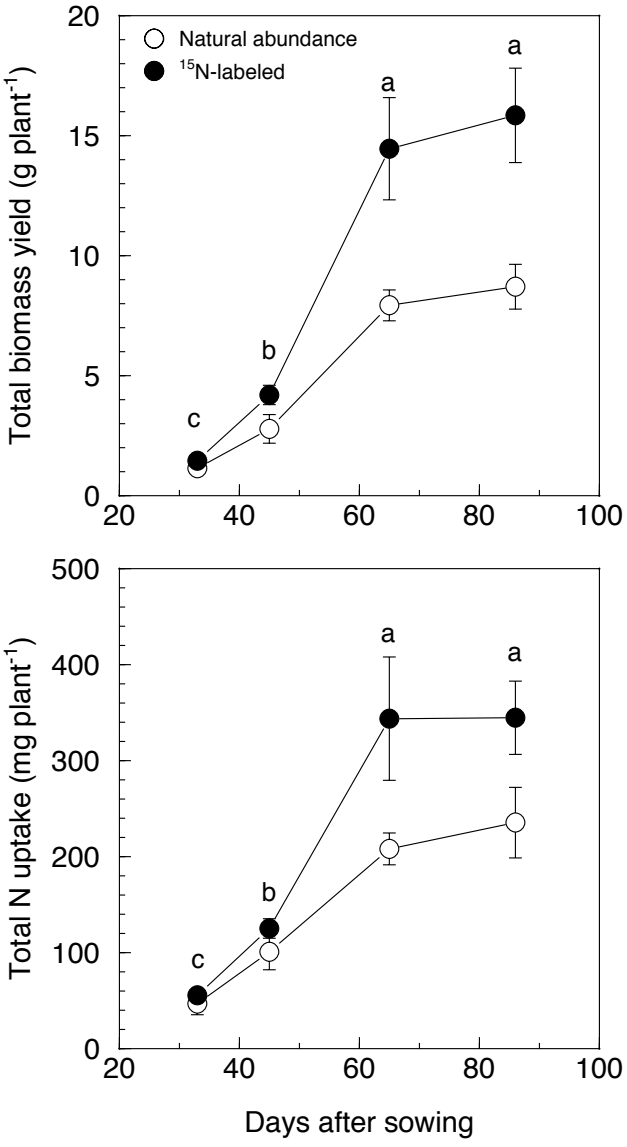

Fig. 3.4. Total dry matter biomass (top) and plant N (bottom) of pea harvested at the vegetative stage (n=6), flowering (n=6), pod filling (n=5), and maturity (n=4) either supplied with $^{15}N_2$ or grown under natural abundance conditions (n=3). Similar letters above means indicate no significant difference between growth stages for both ^{15}N-labeled and control plants according to Tukey's HSD (*P*>0.05).

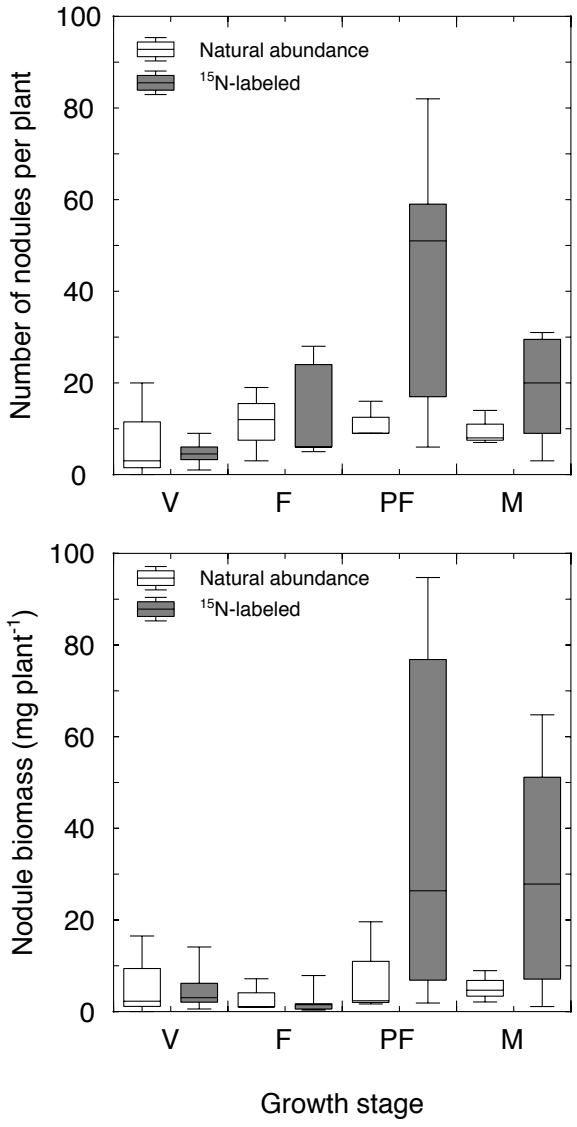

Fig. 3.5. Number of nodules per nodulated pea plant (top panel) and nodule biomass (bottom panel) harvested at the vegetative stage (V, n=4), flowering (F, n=5), pod filling (PF, n=5), and maturity (M, n=4) that were supplied with $^{15}N_2$ or grown under natural abundance conditions (n=3). The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum, respectively.

3.5.3. Plant ^{15}N and Ndfa

Nitrogen fixation was active in some plants, as is indicated by ^{15}N enrichment in plant components (Fig. 3.6). In particular, nodules were significantly more enriched in ^{15}N compared to controls (P=0.002). However, atom% ^{15}N data were variable for other plant components and no statistical differences were detected. Nevertheless, it is evident that fixation of $^{15}N_2$ did occur, with atom% ^{15}N values frequently exceeding 0.4000 for components of individual plants (Fig. 3.6). Since plant atom% ^{15}N values were low relative to soil atmospheric atom% ^{15}N, total %Ndfa was low, ranging from 0.2 to 12.3% at pod filling and 0.7 to 17.8% at maturity (Fig. 3.7). The low N fixation in this system was partially attributable to low nodulation—there was a significant linear relationship between nodule biomass and the amount of fixed ^{15}N in pea (r^2=0.83, P<0.001, Fig. 3.8).

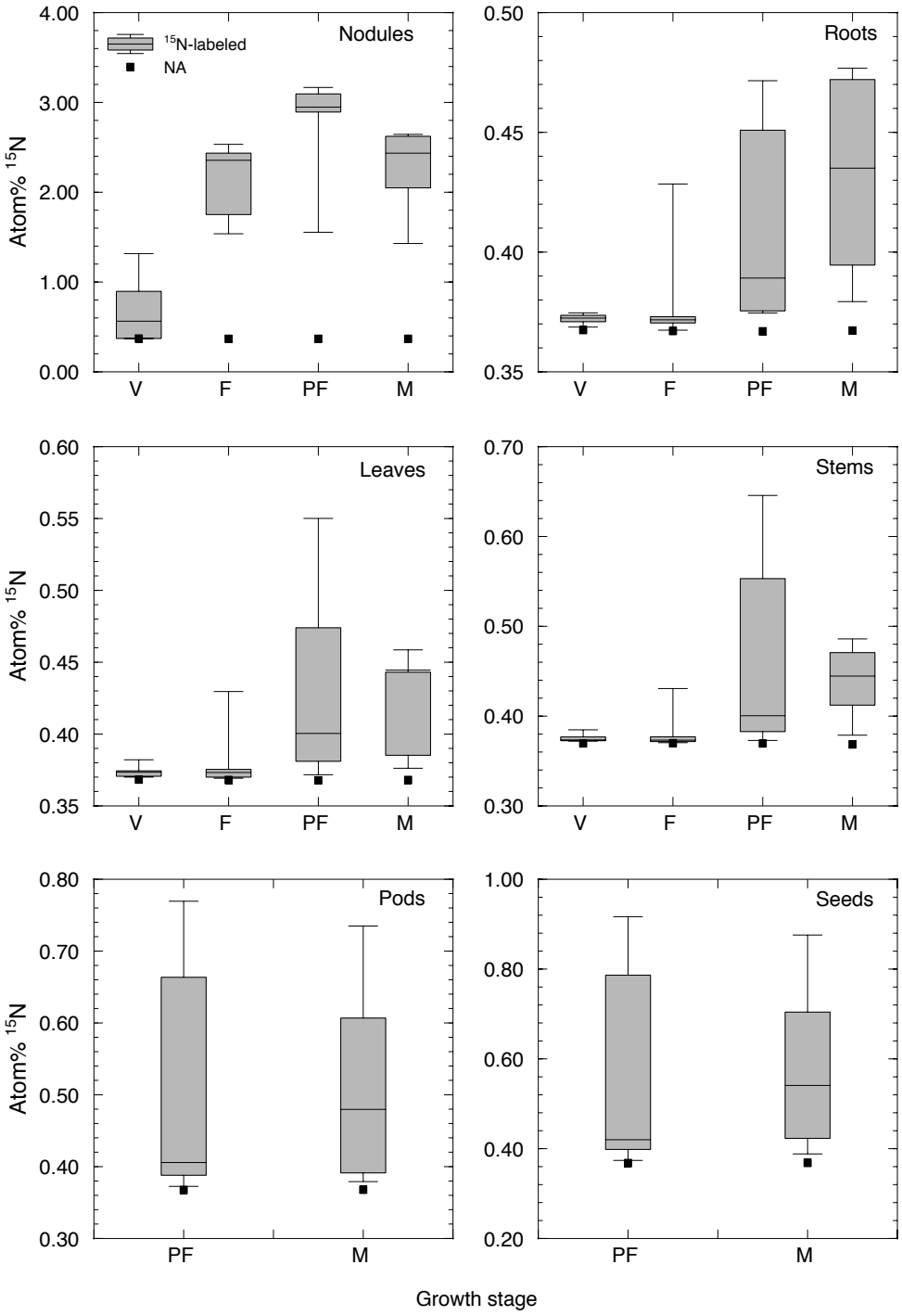

Fig. 3.6. Atom% ^{15}N values (not in excess) for plant components of nodulated pea harvested at the vegetative stage (V, n=4), flowering (F, n=5), pod filling (PF, n=5), and maturity (M, n=4) either supplied with $^{15}N_2$ or grown under natural abundance (NA) conditions (n=3). Standard deviations are plotted for means of the natural abundance plants, but are too small to be visible. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum, respectively.

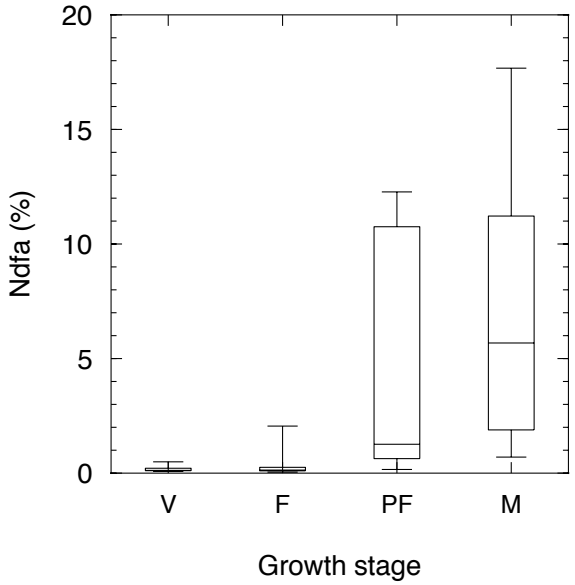

Fig. 3.7. Nitrogen derived from the atmosphere (%Ndfa) calculated on a whole-plant basis for nodulated pea harvested at the vegetative stage (V, n=4), flowering (F, n=5), pod filling (PF, n=5), and maturity (M, n=4) supplied with $^{15}N_2$. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum, respectively.

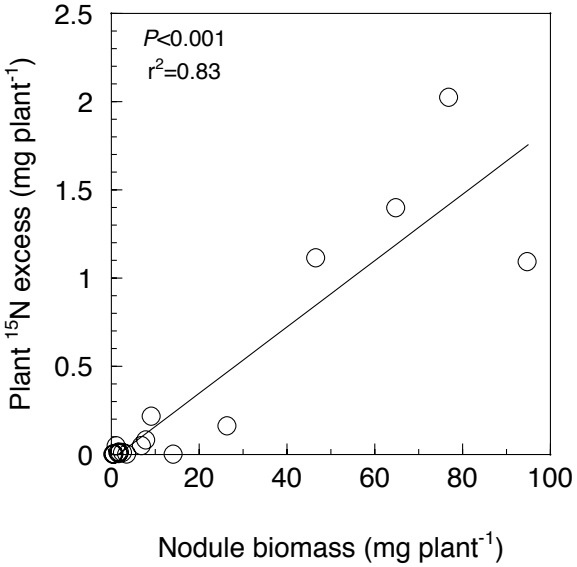

Fig. 3.8. Relationship between nodule biomass and total ^{15}N excess in plant biomass of nodulated pea supplied with ^{15}N using a continuous atmospheric $^{15}N_2$ approach under controlled conditions in a greenhouse.

3.5.4. Rhizosphere ^{15}N and NdfR

Atom% ^{15}N values in the rhizosphere of plants exposed to $^{15}N_2$ were not significantly greater than in the rhizosphere of control plants. However, rhizosphere atom% ^{15}N values were more variable for $^{15}N_2$-exposed compared to control plants, and ^{15}N enrichment was likely for some individual plants (Fig. 3.9). There were significant relationships between atom% ^{15}N excess values in the rhizosphere and measures of nodulation, including nodule biomass, nodule number, and atom% ^{15}N excess of nodulated roots (Fig. 3.10).

Nitrogen derived from rhizodeposition of nodulated pea in the rhizosphere soil averaged between 2.8 and 3.6% across all growth stages. However, %NdfR varied greatly within each growth stage—with the widest range (0 to 8.3%) observed at pod filling (Fig. 3.11). In addition, the partitioning of total plant-assimilated ^{15}N to the rhizosphere varied considerably, with the highest release (4.3%) observed for any individual plant occurring at pod filling (Fig. 3.11).

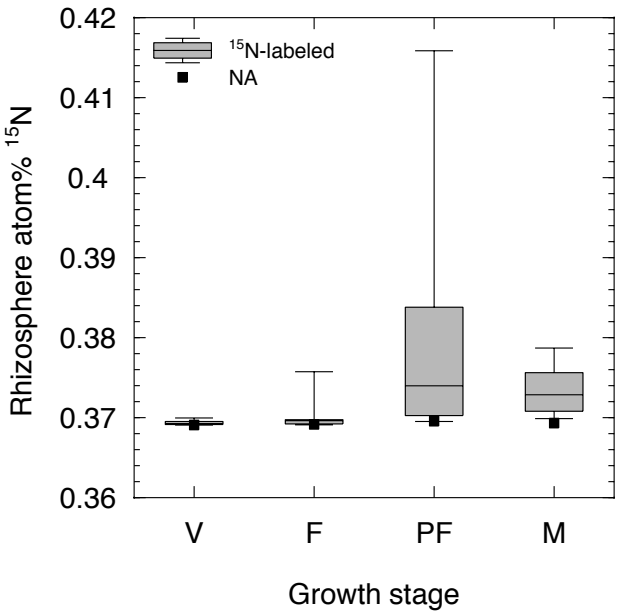

Fig. 3.9. Atom% ^{15}N values (not in excess) in rhizosphere soil of nodulated pea harvested at the vegetative stage (V, n=4), flowering (F, n=5), pod-filling (PF, n=5), and maturity (M, n=4) either supplied with $^{15}N_2$ or grown under natural abundance (NA) conditions (n=3). Standard deviations are plotted for natural abundance means, but are too small to be visible. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum, respectively.

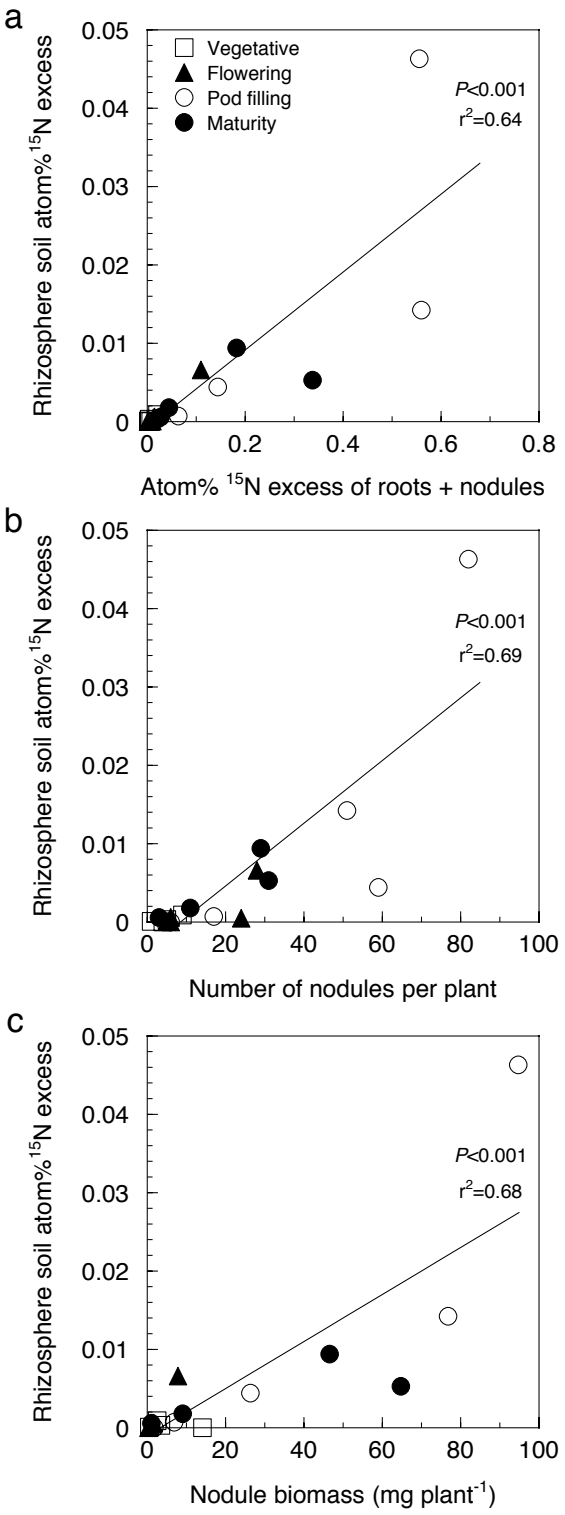

Fig. 3.10. Relationship between atom% ^{15}N excess in roots and nodules (a), number of nodules per plant (b), and nodule biomass (c) and atom% ^{15}N excess in rhizosphere soils of nodulated pea supplied with ^{15}N using a continuous atmospheric $^{15}N_2$ method under controlled conditions in a greenhouse.

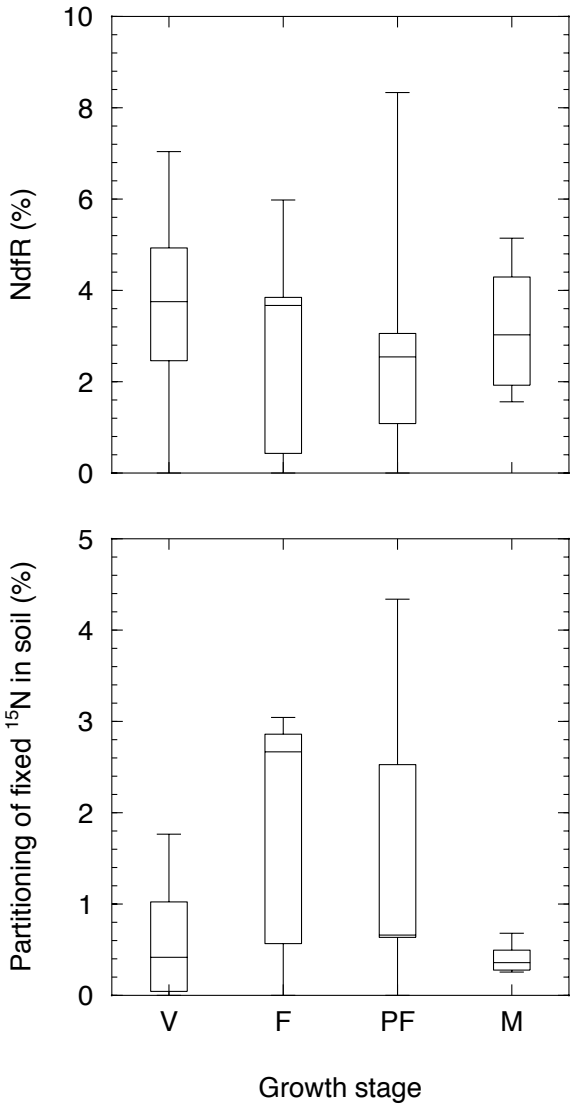

Fig. 3.11. Nitrogen derived from rhizodeposition (%NdfR) and partitioning of fixed-^{15}N (%) in the rhizosphere soil of nodulated pea harvested at the vegetative stage (n=4), flowering (n=5), pod filling (n=5), and maturity (n=4) and supplied with $^{15}N_2$. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum, respectively.

3.6. Discussion

3.6.1. Soil atmosphere ^{15}N enrichment

Maintaining a constant ^{15}N enrichment in the soil atmosphere is a common challenge in $^{15}N_2$-labeling studies (McNeill et al., 1994; Mohr et al., 1998; Russelle et al., 1994; Verburg et al., 2004), and the present study was no exception. Indeed, leaks in the closed-loop gas circulating system prevented high levels of ^{15}N enrichment from being attained and resulted in fluctuations in ^{15}N over the course of the labeling experiment. Nevertheless, variability in atom% ^{15}N in the soil atmosphere among pots was relatively low, which indicates that each plant was exposed to a similar level of ^{15}N at any given time.

3.6.2. Plant growth, N accumulation, and N_2 fixation over time

Nodule biomass is related to N_2 fixation activity (Voisin et al., 2003). Indeed, we found a significant linear relationship between nodule biomass and the amount of fixed ^{15}N in pea. Nitrogen fixation, as evidenced by ^{15}N-enrichment, was active in the nodules present at flowering, pod filling, and maturity; and to a lesser extent during the vegetative stage. Despite this, however, ^{15}N enrichment of the plant parts was generally low, with a high degree of variability. As a result, Ndfa was low, with a maximum value recorded at maturity (17.8%). Pea often acquires more than 50% of its N from fixation. Indeed, pea grown in the field at the same site from which the soil used in this study was collected acquired between 14 and 59% of its N from fixation (Knight, 2012). The lowest N_2 fixation values in the field corresponded to pea grown in monoculture, while higher N_2 fixation consistently occurred for pea grown in more diverse crop rotations (Knight, 2012). As well, Matus et al. (1997) reported that Ndfa in pea seed was 64% averaged over conventional and zero tillage treatments; Stevenson and van Kessel (1996a) reported Ndfa values of 74 to 81% at three sites across the Canadian prairies. However, N_2 fixation has also been shown to vary markedly across a landscape, ranging between zero and almost 100% as determined by both natural abundance and isotope dilution methods (Androsoff et al., 1995). Nodulation was low and variable over the course of the experiment—with the greatest number of nodules occurring at pod filling (82 nodules plant^{-1}). Other published reports have demonstrated high rates of N_2 fixation by plants nodulated to the same extent as those reported here. For example, in five pea cultivars with only 14 to 21 nodules plant^{-1}, the

percentage of fixed N after six weeks of growth ranged between 87.2 and 91.3% (Abi-Ghanem et al., 2011). Clayton et al. (2004) reported that even when the number of nodules was low (<10), N_2 fixation contributed 8 to 71% of total N accumulated in pea grown in the field. Nevertheless, given the positive relationship between nodulation and plant ^{15}N accumulation in the present study, the low N_2 fixation is partially attributed to low nodulation.

High concentrations of available soil-N can inhibit nodule formation and nitrogenase activity (Naudin et al., 2011; Waterer and Vessey, 1993). Indeed, N_2 fixation is tightly linked to overall plant N demand as well as source-sink relationships within the plant. Nitrogen fixation was active in nodules as indicated by ^{15}N enrichment, yet the reliance of pea on N fixation for its N nutrition was relatively limited, suggesting that the availability of soil N and the N demand of the plant may have regulated the degree of N_2 fixation (Schubert, 1995). Voisin et al. (2002b) determined that, for pea grown in the field, N_2 fixation was not initiated unless soil NO_3^- concentrations dropped below 56 kg N ha^{-1}, and that the %Ndfa in the plant at harvest decreased in a linear fashion as the soil NO_3^- concentration at time of sowing increased. Similar results were reported by Jensen (1987). These results certainly suggest that, in the present study, the high soil NO_3^- concentration at the time of sowing could have inhibited nodulation and subsequent N_2 fixation. Indeed, the plants did not show any visible signs of nutrient stress—they were green and robust throughout the vegetative and reproductive stages. It is well known that a variety of edaphic, climatic and biotic factors can limit N_2 fixation (Dita et al., 2006; O'Hara et al., 1988; Schubert, 1995; Zahran, 1999). Therefore, the fact that this study was conducted under controlled conditions in a greenhouse effectively eliminated many of these constraints (e.g., temperature, light availability, competition, and insects). Moreover, water was not limited, as the plants were monitored daily and supplied with water as needed, suggesting that other factors, particularly NO_3^- availability, were influencing N_2 fixation.

Nitrogen fixation in pea has been observed to peak between flowering and early pod filling (Fischinger and Schulze, 2010; Jensen, 1987; Voisin et al., 2002b; Voisin et al., 2003), and then decrease sharply thereafter (Jensen, 1987; Voisin et al., 2002b; Voisin et al., 2003). The high atom% ^{15}N in nodules during pod filling supports these observations (Fig. 3.6). The decline in N_2 fixation may be partially attributed to decreased growth rate, leaf senescence, C competition between nodules and reproductive organs, and the onset of nodule senescence occurring near physiological maturity (Schubert, 1995; Schulze, 2004; Voisin et al., 2002b). Nodule biomass

tended to be lower at maturity than at pod filling (P=0.076). Therefore, the reduction in atom% ^{15}N excess in the soil atmosphere at late pod filling due to a large gas leak likely did not affect the assimilation of ^{15}N from fixation to any great extent; i.e., by this time the plant had already assimilated most of its N from fixation. Moreover, total biomass and N accumulation did not increase between pod filling and maturity, indicating that N was not being acquired from the soil or from fixation; but rather that N was being transferred into the seed from existing sources within the plant (i.e., the leaves, stems, and pods). That is, endogenous N was transferred from the leaves to meet the increasing demand for N during seed development (Schiltz et al., 2005), as opposed to being supplied by fixation (Fischinger and Schulze, 2010).

Nitrogenase is extremely sensitive to oxygen (Schulze, 2004); yet, despite this sensitivity, oxygen must be present to meet the oxidative requirements of the energy intensive N_2 fixation process (Mylona et al., 1995). Legume nodules are therefore highly adaptive (Schulze, 2004), and internal O_2 can be regulated by an O_2 diffusion barrier (Minchin, 1997). As well, N_2 fixation in pea has been shown to be unaffected at both high (30%) and low (10%) external O_2 concentrations (Minchin et al. 1985; reviewed in Schulze, 2004). In the present study, the O_2 concentration in the soil atmosphere was maintained between 15 and 20%, with an average of 17.9% (s.d.=3.4%) over the course of the experiment. In similar $^{15}N_2$ labeling studies, soil O_2 concentrations ranged from 16 to 23% (Mohr et al., 1998) and 14 to 22% (Bremer et al., 1995) without any reported adverse effect on symbiotic or associative N_2 fixation, respectively. Thus, the oxygen concentrations that the pea nodules were exposed to within the closed-loop $^{15}N_2$-labeling system were unlikely to have negatively influenced N_2 fixation.

There was no evidence to suggest that the $^{15}N_2$ labeling system created conditions (e.g., soil moisture, O_2 concentration) that adversely affected nodulation of pea roots. Indeed, nodule number and biomass of the ^{15}N-labeled plants was similar to, and often exceeded that of the control plants—particularly as they matured. Likewise, total plant biomass and N accumulation by pea was generally greater in plants grown in the $^{15}N_2$ atmosphere, likely due to differences in light exposure and soil moisture. It must be stressed that the low N fixation was not due to the $^{15}N_2$ labeling system; i.e., based on nodule biomass and number (Fig. 3.5), N_2 fixation was also likely limited in the natural abundance control plants.

3.6.3. Transfer of fixed N to rhizosphere soil

Enrichment of ^{15}N in the rhizosphere soils of individual plants did occur, indicating the transfer of fixed-^{15}N from the roots to the soil. Although higher root ^{15}N enrichments than were achieved in this study would provide more accurate estimates of N rhizodeposition (Schmidtke, 2005a), Mahieu et al. (2007) calculated reliable estimates of N rhizodeposition from pea roots with relatively low ^{15}N enrichment (e.g., 0.24 atom% ^{15}N excess) using a split-root labeling technique. The weighted average ^{15}N enrichment of roots plus nodules was 0.0086, 0.0299, 0.2673, and 0.1480 atom% ^{15}N excess at the vegetative stage, flowering, pod filling, and maturity, respectively—and there was a significant relationship between the ^{15}N enrichment in the rhizosphere soil and the ^{15}N enrichment in the roots and nodules of the pea plants. Nitrogen-15 enrichment of the rhizosphere soil also was significantly related to the number of nodules present on the roots (on a per plant basis) and nodule biomass. Nodule number is controlled by the plant based on a negative feedback regulatory system—the plant will stop growing nodules when it is no longer energetically favorable (Oka-Kira and Kawaguchi, 2006). Therefore, in the absence of factors that may significantly limit N_2 fixation, ^{15}N enrichment of rhizosphere soil is expected to increase with nodule biomass and nodule number.

Nitrogen derived from rhizodeposition of nodulated pea in the rhizosphere soil ranged from zero to 8% across all growth stages. This amounted to between 1.38 and 2.76 mg N plant^{-1}—or less than 3% of total plant N (not shown). Using shoot ^{15}N labeling methods, N rhizodeposition ranged widely—from 2.4 to 36.4% of total plant N in pea at maturity (Mahieu et al., 2009a; Mayer et al., 2003b; Wichern et al., 2007a; Wichern et al., 2007b). In alfalfa, NdfR comprised 12% of total plant N when nodulated roots were exposed to $^{15}N_2$ (Mohr et al., 1998). If N_2 fixation had been higher in my system, root and nodule atom% ^{15}N enrichment would have been higher and ^{15}N likely would have been detected in the bulk soil as well, thus increasing the total belowground input of N detected.

Nitrogen-15 detected in the rhizosphere is indicative of the release of fixed-N from the plant to soil. Between zero and 4.3% of the total ^{15}N fixed by pea was released to the rhizosphere throughout growth. Indeed, the data were extremely variable within each growth stage, with CVs ranging from 47 to 126% (Fig. 3.11), with the maximum value for any individual plant occurring during pod filling. At alfalfa termination, 12% of ^{15}N in the plant-soil system was recovered in the soil (Mohr et al., 1998), similar to that for common bean (12 to 18%) (Ruschel et al., 1979).

Conducting further studies that induce a range of N_2 fixation rates may help to elucidate some of the questions that remain due to the low N_2 fixation in this system—that is, does the amount and the proportion of fixed N released to soil increase at rates of N_2 fixation commonly reported in the field?

I did not include a non-nodulating isoline or non-legume plant in the experimental design due to logistical constraints and, therefore, cannot definitively rule out the input of free living or associative N_2 fixation to the ^{15}N enrichment detected in the rhizosphere soils. However, using similar $^{15}N_2$ labeling at 20 atom% ^{15}N in the soil atmosphere, Bremer et al. (1995) reported that associative N_2 fixation with wheat contributed only 30 g N ha^{-1} to the soil. Moreover, the significant relationship between nodule number and rhizosphere soil ^{15}N enrichment and between nodule biomass and plant ^{15}N enrichment provides evidence that the ^{15}N in the rhizosphere soil was a result of the release of symbiotically fixed-N to soil from the roots and nodules rather than associative N_2 fixation.

3.7. Conclusion

The release of fixed N to soil was minimal due to low rates of N_2 fixation. This highlights the fact that N_2 fixation is not always guaranteed when pulse crops are grown. This is especially true in soils with adequate soil N availability, in which case the crop may acquire most of its N from the soil and N_2 fixation will be low. However, even at very low rates of N_2 fixation, fixed N was released from the nodulated pea roots into rhizosphere soil. When higher nodulation occurs, rates of N_2 fixation are expected to be higher and therefore the amount of fixed N released to the rhizosphere soil is expected to be greater. Although there was no evidence to suggest that the $^{15}N_2$ labeling system was responsible for the low N_2 fixation observed in my study, the expense and technical difficulties in maintaining the continuous $^{15}N_2$ labeling limit its use. Therefore, alternative methods to assess N_2 fixation and N rhizodeposition such as isotope dilution and shoot ^{15}N labeling, respectively, will continue to play a significant role in studies examining the flow of N in pulse cropping systems.

4. TEMPORAL DYNAMICS OF NITROGEN RHIZODEPOSITION BY PEA AS DETERMINED BY ^{15}N LABELING

4.1. Preface

Given the technical difficulties and costs associated with atmospheric $^{15}N_2$ labeling, shoot-^{15}N labeling methods have been developed to estimate the amount of root N that is released to soil during legume growth. Nitrogen fixation varies according to plant phenology and, therefore, it is likely that the release of N compounds from N fixing plants may also vary. In this greenhouse experiment, N rhizodeposition in rhizosphere and bulk soils was quantified at the vegetative stage, at flowering, and at physiological maturity of pea supplied with ^{15}N-urea using the cotton-wick ^{15}N labeling technique. The objectives of this study were to determine how belowground partitioning of plant N in pea varies over the growing season as well as to determine the cumulative input of root-derived N to soil over time.

4.2. Abstract

Assessing the contribution of symbiotically fixed N_2 to soil from pulse crops necessitates a full accounting of the total crop residue N remaining in the field after seed harvest. Belowground N, including root and rhizodeposit N, comprise an important component of this total plant N balance—without it, the N input to soil is underestimated. Under controlled conditions in a greenhouse, N in intact roots and N rhizodeposition were quantified in field pea (*Pisum sativum* L.) using the cotton-wick ^{15}N labeling technique. Plants were supplied with ^{15}N on a continuous basis and harvested at the vegetative stage (9-leaves unfolded), flowering, and maturity. As the plants aged, the ^{15}N enrichment in the rhizosphere soil decreased whereas that in the bulk soil increased, suggesting that N released as root exudates comprised a more important proportion of N rhizodeposition in plants at the early vegetative stage compared to mature plants. In mature plants, N rhizodeposition was comprised predominantly of N associated with root turnover. The contribution of N rhizodeposition recovered in soil to the total plant N balance decreased from 17.9% at the vegetative stage harvest, to 12.3% at flowering, and finally to 7.6% at maturity.

However, the total amount of root-derived N released to soil by pea increased with plant development. Belowground N, including N rhizodeposition and N in intact roots contributed 11.3% to the total plant N balance of mature pea.

4.3. Introduction

Nitrogen (N) fertilizer represents the greatest energy input into agricultural systems, contributing more than 50% of the total carbon footprint (Gan et al., 2011). Annual grain legumes (pulse crops) can satisfy a portion of their N requirement through symbiotic N_2 fixation and thus can play an important role in efforts to reduce overall N fertilizer use in Prairie cropping systems. Furthermore, pulse crops can provide a net input of N to the soil if N_2 fixation is higher than the seed N requirement (Walley et al., 2007); thereby increasing the N supplying power of the soil (i.e., relative to soils in crop rotations that do not include pulse crops) and reducing N fertilizer requirements for subsequent crops (van Kessel and Hartley, 2000). The N contained in roots and rhizodeposits is an important component of the total input of symbiotically fixed N to soil from pulse crops (Herridge et al., 2008; Walley et al., 2007). In addition, assessment of belowground N (BGN) inputs from pulse crops can be used to predict the supply of legume-derived N to succeeding crops (Mayer et al., 2003a; Russell and Fillery, 1996a) and understand its influence on N turnover (Mayer et al., 2004). Until recently, assessment of belowground inputs of N from legumes has been limited and estimates reported in the literature are variable, ranging from 14 to 74% of total plant N (Wichern et al., 2008).

Accumulation of root N over the growing season can be fairly dynamic, such that the amount of N in roots at the end of the growing season may not represent the total N accumulated in roots over the course of the growing season. For example, Gan et al. (2010b) reported that root N peaked at 25 kg N ha^{-1} (0–100 cm depth) at late flowering in pea. However, only 16 kg N ha^{-1} was recovered in pea roots at maturity—comprising 11% and 22% of the total crop and crop residue N balance, respectively (Gan et al., 2010b). In the same study, root biomass decreased 30% between flowering and maturity, indicating a loss of root mass to the soil, presumably as dead roots (Gan et al., 2009c). The reduction in root biomass and root N between flowering and maturity suggests that there was a N input to the soil as dead roots decayed prior to crop harvest. However, a portion of the N also was likely reallocated from roots to shoots between flowering and maturity (Schiltz et al., 2005) and, as a result, the reduction in root N between growth stages

does not equate to the amount of N input to soil from dead roots. Moreover, N released from roots of living plants throughout the growing season (i.e., N rhizodeposition) has been reported to comprise as much as 36.4% of total N in pea at maturity (Wichern et al., 2007a). Therefore, the cumulative N released from roots over the course of the growing season cannot be adequately accounted for when relying only on physical recovery of roots at crop maturity.

Nitrogen-15 labeling techniques have been developed to address the inadequacy of relying on physical root recovery to determine the total belowground N (BGN) contribution to the N balance of pulse crops. Indeed, the N released by plant roots from a variety of grain and forage legumes has been estimated using atmospheric $^{15}N_2$ labeling (McNeill et al., 1994; Russelle et al., 1994); split-root techniques (Jensen, 1996c; Mahieu et al., 2009b; Sawatsky and Soper, 1991); and leaf and stem labeling (Khan et al., 2002b; Lam et al., 2012; López-Bellido et al., 2011; Mayer et al., 2003b; Russell and Fillery, 1996b; Wichern et al., 2007a; Yasmin et al., 2010). Atmospheric labeling provides the most direct measure of the release of fixed-N_2 to soil from legumes, but is expensive and technically challenging. Split-root ^{15}N labeling involves splitting half the root system of the growing plant in a ^{15}N-fertilized growth medium and the other half in a receiving soil compartment in which rhizodeposition is determined. Although ^{15}N is assimilated naturally via root uptake, split-root labeling is difficult to apply in the field, drastically disturbs the root system, and N rhizodeposition is accounted for only half the root system (Wichern et al., 2008). Leaf and stem labeling involves introducing a highly enriched solution of either ^{15}N-urea or $^{15}NO_3^-$ directly into the leaf or stem of the plant. Stem labeling techniques, in particular, have been widely used, probably due to the relative ease of installation of the ^{15}N-labeling apparatus as well as their potential applicability to field studies (Wichern et al., 2007a).

The contribution of N rhizodeposition to total plant N in mature pea estimated using the cotton-wick stem ^{15}N-labeling technique has been reported as 2.7% (Mahieu et al., 2009a), 12.8% (Mayer et al., 2003b), 14.4% (Arcand et al. 2013), 29.2% (Wichern et al., 2007b), and 36.4% (Wichern et al., 2007a). Whereas the wide range in results is likely attributable to differences in plant and edaphic factors (Wichern et al., 2008), it also reflects variations in the frequency and duration of the ^{15}N supply (Mahieu et al., 2009a). For example, using a continuous ^{15}N-labeling approach, Mahieu et al. (2009a) reported relatively low contributions of N rhizodeposition to the total plant N balance (between 2.7 and 5.5%). Conversely, the high

values of N rhizodeposition of mature pea obtained by Wichern et al. (2007a; 2007b) were determined for plants receiving only two doses of ^{15}N during the early stages of plant growth. Infrequent supply of ^{15}N over the course of plant growth may result in overestimation of N rhizodeposition due to ^{15}N dilution by root uptake of ^{14}N from the soil or N_2 fixation—particularly if root growth and N uptake increases between completion of ^{15}N labeling and plant harvest (Rasmussen, 2011). This is because N rhizodeposition is estimated based on the ratio of atom% ^{15}N excess of the soil and that of the roots (Janzen and Bruinsma, 1989), and assumes uniform and constant root ^{15}N enrichment over crop growth (Mayer et al., 2003b). Therefore, any reduction in root ^{15}N enrichment without a concomitant release of ^{15}N from roots to soil can erroneously increase calculations of N rhizodeposition. Conversely, successive labeling can increase the atom% ^{15}N excess of the roots (Mayer et al., 2003b), as well as the distribution of ^{15}N throughout the plant (Russell and Fillery, 1996b). Consequently, providing a constant supply of ^{15}N to plants can improve the accuracy of N rhizodeposition estimates (Mahieu et al., 2009a), particularly if it is synchronized with plant N demand (Janzen and Bruinsma, 1989). Previous shoot-labeling studies that examined N rhizodeposition at different growth stages of pea used either single, double, fortnightly pulses, or applied the label at specific growth stages (Mahieu et al., 2007; Wichern et al., 2007a; Wichern et al., 2007b). However, estimates of the cumulative input of N from pea roots to soil at different plant growth stages are incomplete. For example, using continuous ^{15}N labeling, Mahieu et al. (2009a) determined N rhizodeposition only during the later growth stages. As well, there are few studies of N rhizodeposition in soil during early growth stages (Wichern et al., 2007b); though ^{14}C labeling studies indicate a decreased partitioning of C to rhizodeposition as plants age (Jones et al., 2009).

The primary objective of this study was to quantify N rhizodeposition at the vegetative stage, early flowering, and physiological maturity of field pea under greenhouse conditions. This was accomplished by providing a continuous supply of ^{15}N using the cotton-wick ^{15}N-labeling method throughout the growth period. A secondary objective was to compare the partitioning of ^{15}N within aboveground and belowground plant parts and the distribution of ^{15}N in rhizosphere and bulk soils of the ^{15}N-labeled pea plants at various growth stages. Nitrogen rhizodeposition was defined as the root-derived N remaining in the soil, not including visible roots and root fragments.

4.4. Materials and Methods

4.4.1. Soil preparation and planting

Pots constructed of polycarbonate tubing (12 cm dia., 30 cm deep) were packed with a soil-sand mixture to a bulk density of 1.3 g cm^{-3}. The soil, an Orthic Dark Brown Chernozem collected from an agricultural research field site (Scott, SK), was air-dried, sieved (4 mm) to remove any rocks, and mixed with silica sand in a 1:1 ratio by weight. The resulting soil-sand mixture had a pH of 5.6 (1:2 soil to H_2O), was sandy loam in texture, contained 1.3 g total N kg^{-1}, 77.3 mg NO_3^--N kg^{-1}, 103.1 mg P kg^{-1}, 734.3 mg K kg^{-1}, and 12.3 mg S kg^{-1}. Five pea seeds (cv. CDC Meadow), inoculated with *Rhizobium leguminosarum* (Nodulator® sterilized peat-based, Becker Underwood, Saskatoon, SK) at 1.5 times the recommended rate, were sown into each pot and thinned to one plant per pot following germination. Plants were watered regularly with deionized water to maintain approximately 80% field capacity determined gravimetrically. The pots were arranged on a greenhouse bench as a completely randomized design with nine replicates for each harvest period (vegetative, flowering, maturity) for the ^{15}N-labeled plants. Four replicate non-labeled natural abundance control plants were included for each harvest period and grown on an adjacent greenhouse bench.

4.4.2. ^{15}N Labeling Method

Pea plants were supplied with ^{15}N-enriched urea using the cotton-wick method (Russell and Fillery, 1996b), with the wicks inserted directly into the stem. Labeling frequency was adapted from the continuous ^{15}N labeling approach described by Mahieu et al. (2009a), while labeling dosage was based on plant N demand as outlined by Mayer et al. (2003b), and determined in a preliminary experiment. Briefly, a 0.5 mm hole was drilled into the stem of the plant, between the second and third nodes, and a cotton thread was fed through the hole in the stem using a thin needle. The cotton thread was protected on either side of the plant stem with silicone tubing (0.76 mm i.d. × 4 cm length), which was sealed to the stem of the plant using plasticine. The ends of the silicone tubing, including the enclosed cotton thread were fed through the cap of a 2 mL vial and the thread immersed in a 0.4% (w/v) ^{15}N-enriched urea solution (99.2 atom% ^{15}N). The cap of the vial consisted of a Teflon coated silicone septum with a hole small enough to allow for the silicone tubing and the thread to feed through, but provided sufficient seal to

minimize evaporative loss. A needle and syringe were used to replenish the ^{15}N-urea solution in small doses (0.10–0.57 mL) over the course of the experiment (at one to five day intervals) by injecting the urea through the septum of the cap. When plant uptake of ^{15}N appeared to slow significantly (i.e., it took longer than three days to completely absorb the solution in the vial), the wick systems were reinstalled. To maximize ^{15}N urea solution uptake from the wick into the plant stem, 0.40 mL of deionized water was added to each vial after the plant had taken up the last addition of ^{15}N urea solution. Wicks and vials of the ^{15}N labeling apparatus were removed from the plant stem at harvest. Labeling commenced 18 days after sowing (DAS; corresponding to 4 leaves unfolded) for all plants and continued until 25, 41, and 72 DAS for plants harvested at the vegetative stage (32 DAS; corresponding to 9 leaves unfolded), early flowering (55 DAS), and physiological maturity (96 DAS), respectively.

4.4.3. Soil and plant sample preparation and analysis

Aboveground plant components were separated into leaves, stems, pods, and seed, and dried at 60°C. Pots containing soil and intact roots were stored at 2°C until the roots could be removed from the soil. Intact roots and visible root fragments were removed from the soil using a 2-mm sieve and tweezers. Soil from which roots were removed was considered bulk soil; soil adhering to the roots was considered rhizosphere soil. Roots were washed on a 0.5 mm sieve with deionized water. Nodules were removed from the roots and were analyzed separately. The soil-water slurry from root washing was collected and dried in an oven at 75°C to recover the rhizosphere soil. Soil and plant samples were finely ground in a ball mill and were weighed and analyzed for N concentration (%) and $\delta^{15}N$ using an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific Inc., Waltham, MA) coupled to an elemental analyzer (Costech ECS4010, Costech Analytical Technologies, Inc., Valencia, CA).

4.4.4. Calculations

The percentage of soil N derived from rhizodeposition (% NdfR) was calculated according to the equation (Janzen and Bruinsma, 1989):

$$\%\text{NdfR} = \frac{\text{atom\% } ^{15}\text{N soil} - \text{atom\% } ^{15}\text{N background A}}{\text{atom\% } ^{15}\text{N roots} - \text{atom\% } ^{15}\text{N background B}} \times 100 \qquad [4.1]$$

where background-A and background-B are the natural abundance atom% ^{15}N values in the soil and the roots of non-labeled plants, respectively (Schmidtke, 2005b). Separate %NdfR values were calculated for the rhizosphere and bulk soils based on their unique atom% ^{15}N values from the ^{15}N-enriched and non-labeled treatments. Quantities of NdfR in the bulk and rhizosphere soils were calculated by multiplying %NdfR by the total soil N (mg pot^{-1}) in each respective soil fraction. These calculations represent net N rhizodeposition and, as such, do not consider reabsorption of N by the plant. Assumptions of the calculation include uniform distribution of ^{15}N within the root system, constant ^{15}N enrichment over the growth cycle, and equal ^{15}N enrichment of the recovered (visible) roots and rhizodeposits (Janzen and Bruinsma, 1989; Mayer et al., 2003b).

Nitrogen-15 distribution ratios were calculated for the aboveground plant component (i.e., leaves, stems, seeds combined), roots, and nodules. Values greater than 1.0 indicate preferential ^{15}N enrichment, while values less than 1.0 indicate discrimination against ^{15}N enrichment. They are calculated by taking the ratio of the amount of ^{15}N excess in a plant component as a percentage of the total amount of ^{15}N excess in all plant components (% of total ^{15}N recovered) and the amount of N in the same plant component as a percentage of the total amount of N in all plant components (% of total plant N), without taking the soil ^{15}N and N into consideration (Russell and Fillery, 1996b).

4.4.5. Statistics

Statistical analyses were performed using SPSS® Statistics version 20.0 for Mac (IBM Corp., 2011). One-way analysis of variance was performed with growth stage as a fixed effect. Comparison of atom% ^{15}N values between the ^{15}N-labeled treatments and the ^{15}N-natural abundance controls within a plant or soil component were completed using a two-tailed Student's t-test. Comparison of atom% ^{15}N excess values between plant parts was conducted using a paired t-test. Means comparisons were made using Tukey's Honestly Significant Difference test. All tests were declared significant at $P \leq 0.05$.

4.5. Results

4.5.1. Plant biomass

Biomass increased for all plant components between the vegetative stage (32 DAS) and

flowering (55 DAS; Fig. 4.1). Whereas leaf, stem, and nodule biomass remained constant between flowering and maturity (P>0.05), root biomass decreased during the same period (P=0.003; Fig. 4.1). Seed comprised 57% of total plant biomass, representing the majority of total plant biomass at maturity. Roots plus nodules comprised 26.5, 23.5, and 4.9% of total plant biomass at the vegetative, flowering, and maturity growth stages, respectively. At the same growth stages, nodules comprised 5.9, 4.1, and 10.7% of total belowground biomass.

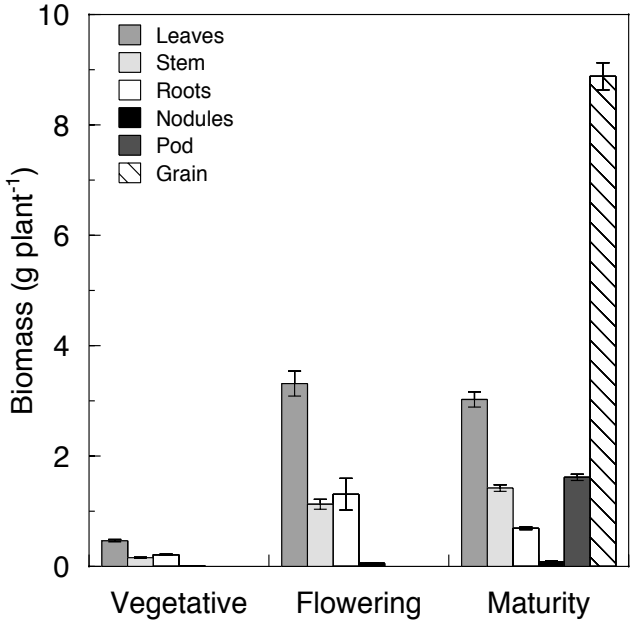

Fig. 4.1. Biomass (g plant^{-1}) of each plant component of pea grown in a greenhouse. Plants were supplied with ^{15}N-urea using the cotton-wick ^{15}N labeling technique and were harvested at the early vegetative stage (32 DAS), flowering (55 DAS), and maturity (96 DAS). Values are means ± standard errors (n=9).

4.5.2. Atom% ^{15}N excess

Atom% ^{15}N excess values varied with growth stage for each plant and soil component (Table 4.1). For both leaves and stem, ^{15}N enrichment was lowest at flowering but did not differ between the vegetative stage and maturity. Root atom% ^{15}N excess values increased as the plants grew; however, this trend was reversed for nodules (Table 4.1). Similar to roots, atom% ^{15}N excess values of bulk soils increased as the plants aged; conversely, atom% ^{15}N excess in the rhizosphere soil was greatest at the vegetative growth stage and tended to decrease with increasing age of the plants—though it did not differ between flowering and maturity.

Nevertheless, at all growth stages, atom% ^{15}N excess values for the rhizosphere soil were about an order of magnitude greater than those for the bulk soil. Soil atom% ^{15}N excess values were significantly greater for plants supplied with ^{15}N-urea compared to the natural abundance controls for both bulk and rhizosphere soils ($P<0.001$, data not shown). The weighted average of roots and nodules used for calculating %NdfR was 1.1589, 1.2591, 1.5013 atom% ^{15}N excess at the vegetative stage, flowering, and maturity, respectively.

4.5.3. ^{15}N distribution ratios within the plant

Aboveground ^{15}N distribution ratios were greater than 1.0 at each growth stage, indicating preferential enrichment of ^{15}N in aboveground plant parts (Fig. 4.2). As ^{15}N labeling proceeded over the growing period, aboveground ^{15}N distribution ratios became closer to unity, decreasing from an average of 1.10 to 1.01 between the vegetative and maturity stages. This trend was reversed in the roots, with ^{15}N distribution ratios increasing from the vegetative stage to maturity (Fig. 4.2). The ^{15}N distribution ratios of nodules were lowest at the vegetative stage, but did not differ between flowering and maturity ($P=0.906$). Distribution of ^{15}N towards roots and nodules was not favoured, with average root ratios ranging from 0.25 to 0.66 and average nodule ratios ranging from 0.44 to 0.57 (Fig. 4.2).

4.5.4. Recovery of ^{15}N and the distribution of recovered ^{15}N within plant and soil

In total, 2.33, 6.38, and 10.49 mg ^{15}N was added as urea to each pea plant harvested at the vegetative growth stage, flowering, and maturity, respectively. Of this added ^{15}N, 75.5, 88.9, and 83.2% was recovered in plant components and soil at the vegetative growth stage, flowering, and maturity, respectively (data not shown). For all belowground components, recovery of ^{15}N was greatest in the bulk soil.

The highest proportion of ^{15}N was recovered in the aboveground plant components, with 91.2, 89.8, and 92.5% of the added ^{15}N recovered at the vegetative stage, flowering, and maturity, respectively (Table 4.1). At maturity, the highest proportion of recovered ^{15}N was allocated towards seed. Partitioning of recovered ^{15}N in the roots was highest at flowering, while in nodules it was highest at the vegetative stage (Table 4.1). Distribution of recovered ^{15}N in the bulk soil averaged between 3.9 and 4.9% from the vegetative stage to maturity, respectively (Table 4.1). In contrast, distribution of recovered ^{15}N in the rhizosphere soil decreased with plant

growth stage—from 1.9 to 0.1% between the vegetative stage and maturity (Table 4.1). There was less rhizosphere soil recovered at maturity compared to flowering due to drier soil conditions at this time, thus influencing the total ^{15}N recovered in the bulk *vs*. rhizosphere soil. Soil ^{15}N comprised 65.5, 58.3, and 67.1% of total belowground ^{15}N recovered (i.e., nodules + roots + soil) at the vegetative stage, flowering, and maturity, respectively.

Table 4.1. ^{15}N enrichment (atom% ^{15}N excess) and distribution of recovered ^{15}N (%) in plant and soil components at the vegetative, flowering, and maturity growth stages of pea grown in a greenhouse and supplied with ^{15}N using the cotton-wick ^{15}N labeling technique.

Growth stage	Plant						Soil	
	Leaves	Stem	Pod	Seed	Roots	Nodules	Rhizosphere	Bulk
					Atom% ^{15}N excess			
Vegetative	4.73 ± 0.19 a†	3.30 ± 0.20 a	–	–	1.02 ± 0.07 b	1.96 ± 0.08 a	0.09 ± 0.01 a	0.001 ± 0.0001 c
Flowering	3.09 ± 0.25 b	2.34 ± 0.19 b	–	–	1.24 ± 0.10 b	1.63 ± 0.12 b	0.07 ± 0.01 ab	0.005 ± 0.0004 b
Maturity	4.68 ± 0.36 a	3.74 ± 0.25 a	2.22 ± 0.10	2.21 ± 0.07	1.52 ± 0.07 a	1.33 ± 0.07 b	0.06 ± 0.00 b	0.008 ± 0.0003 a
P value	<0.001	<0.001	–	–	<0.001	<0.001	0.030	<0.001
					Distribution of recovered ^{15}N (%)			
Vegetative	80.8 ± 1.0 a	10.4 ± 0.9 a	–	–	2.3 ± 0.2 b	0.75 ± 0.09 a	1.91 ± 0.09 a	3.9 ± 0.4 a
Flowering	80.1 ± 0.8 a	9.7 ± 0.3 a	–	–	3.9 ± 0.6 a	0.34 ± 0.05 b	1.10 ± 0.13 b	4.8 ± 0.4 a
Maturity	8.5 ± 1.1 b	1.1 ± 0.1 b	1.8 ± 0.1	81.1 ± 1.1	2.2 ± 0.1 b	0.24 ± 0.04 b	0.09 ± 0.01 c	4.9 ± 0.1 a
P value	<0.001	<0.001	–	–	0.002	<0.001	<0.001	0.076

† Same letters following means ± standard errors (n=9) indicate no significant difference among treatments within each plant or soil component (P>0.05) according to Tukey's HSD test

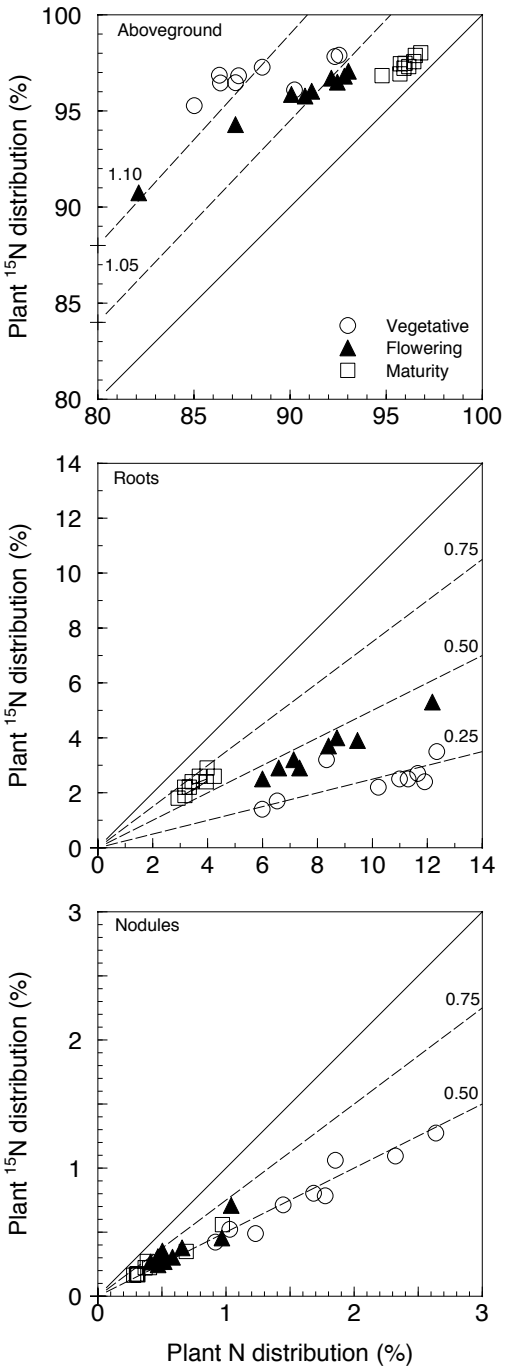

Fig. 4.2. Distribution of plant ^{15}N *vs.* distribution of total plant N in all aboveground plant components, roots, and nodules for pea grown in a greenhouse. Plants were supplied with ^{15}N using the cotton-wick ^{15}N labeling technique and were harvested at the early vegetative stage (32 DAS), flowering (55 DAS), and maturity (96 DAS). The solid line indicates a ^{15}N distribution ratio of 1.00, indicating equal distribution of ^{15}N and N; dashed lines indicate ^{15}N distribution ratios of 0.25, 0.50, 0.75, 1.05, or 1.10.

4.5.5. Total plant N and distribution of root-derived N in soil

Nitrogen accumulation (mg plant^{-1}) in leaves and stems increased between the vegetative stage and flowering, then decreased sharply between flowering and maturity as N was transferred to the seed (Table 4.2). Root N (mg plant^{-1}) increased between the vegetative stage and flowering, but with no significant (P=0.185) change between flowering and maturity—despite a small decrease in root biomass during this same period (Fig. 4.1). The N concentration in roots increased from 1.45% at flowering to 1.85% at maturity (P<0.001; data not shown). Nodule N (mg plant^{-1}) increased between the vegetative stage and flowering and then leveled off between flowering and maturity. Total plant N accumulation (mg plant^{-1}) exhibited a 4.86-fold increase between the vegetative stage (i.e., at 9-leaves unfolded; 32 DAS) and early flowering (55 DAS) and an additional 1.82-fold increase between early flowering and maturity (96 DAS; Table 4.2).

The percentage of N derived from rhizodeposition in bulk soil increased significantly with plant development (P<0.001; Fig. 4.3). In rhizosphere soils, %NdfR was greatest at the vegetative stage (P=0.015) and decreased with plant development, though the difference between flowering and maturity was not significant (P=0.222)—most likely due to the high variability (CV=43%) in the estimates at flowering (Fig. 4.3). Due to the higher ^{15}N enrichment in the rhizosphere soils (Table 4.1), the %NdfR in the rhizosphere exceeded that in bulk soils (Fig. 4.3). However, the amount of NdfR (mg pot^{-1}) was greater in the bulk soils (Table 4.2), due to the greater weight of this soil fraction relative to that of the rhizosphere soil.

The quantity of NdfR recovered in the bulk soil increased during plant development, though the increase was less between flowering and maturity than between the vegetative stage and flowering (Table 4.2). Conversely, though the amount of NdfR recovered in rhizosphere soil at flowering was greater than that recovered at the early vegetative stage (Table 2), there was a sharp decrease in the NdfR recovered in the rhizosphere at maturity.

Nitrogen rhizodeposition comprised 11.9, 10.0, and 7.4% of total plant N in the bulk soil of peas harvested at the vegetative stage, flowering, and maturity, respectively (Table 4.2). Plant-derived N recovered in the rhizosphere soil accounted for a much smaller proportion of total plant N; i.e., 6.0, 2.3, and 0.1% at the vegetative stage, flowering, and maturity, respectively. Together, therefore, total N rhizodeposition accounted for 17.9, 12.3, and 7.6% of total plant N at the vegetative, flowering, and maturity growth stages, respectively. Furthermore, together with the N in roots and root fragments, BGN comprised 27.4, 20.9, and 11.3% of total plant N at these

same growth stages—with N rhizodeposition comprising 59–67% of the total BGN.

Table 4.2. Total plant N (mg plant^{-1}), including N rhizodeposition (NdfR)†, and distribution of plant N (%) at the vegetative, flowering, and maturity growth stages of pea (n=9) grown in a greenhouse and supplied with ^{15}N using the cotton-wick ^{15}N labeling technique.

Growth stage	Plant						Soil		Total	
	Leaves	Stem	Pods	Seed	Roots	Nodules	$NdfR_{RS}$§	$NdfR_{BS}$	Plant only	Plant + NdfR
	mg N plant^{-1}						*mg N pot^{-1}*		*mg N pot^{-1}*	
Vegetative	30.6 ± 1.9 b‡	5.6 ± 0.4 b	–	–	4.1 ± 0.4 b	0.7 ± 0.1 b	3.0 ± 0.2 b	5.8 ± 0.3 c	40.9 ± 1.9 c	51.0 ± 2.7 c
Flowering	153.7 ± 9.1 a	24.7 ± 2.1 a	–	–	19.0 ± 3.7 a	1.3 ± 0.2 a	5.2 ± 0.7 a	22.0 ± 1.7 b	198.7 ± 9.4 b	226.5 ± 14.3 b
Maturity	15.5 ± 1.1 c	2.4 ± 0.2 b	7.2 ± 0.3	321.4 ± 6.4	12.8 ± 0.6 a	1.6 ± 0.3 a	0.5 ± 0.1 c	29.0 ± 1.3 a	361.0 ± 6.5 a	390.1 ± 7.2 a
P value	<0.001	<0.001	–	–	<0.001	0.001	<0.001	<0.001	<0.001	<0.001
	Distribution of total plant N (%)									
Vegetative	61.4 ± 0.9 b	11.2 ± 0.6 a	–	–	8.2 ± 0.7 a	1.4 ± 0.1 a	6.0 ± 0.3 a	11.9 ± 0.9 a	80.1 ± 1.2 c	100
Flowering	68.2 ± 1.0 a	10.9 ± 0.3 a	–	–	8.1 ± 1.1 a	0.5 ± 0.1 b	2.3 ± 0.3 b	10.0 ± 0.9 a	87.5 ± 0.9 b	100
Maturity	4.0 ± 0.3 c	0.6 ± 0.1 b	1.9± 0.1	82.3± 0.5	3.3 ± 0.1 b	0.4 ± 0.1 b	0.1 ± 0.0 c	7.4 ± 0.3 b	92.5 ± 0.3 a	100
P value	<0.001	<0.001	–	–	<0.001	<0.001	<0.001	0.001	<0.001	–

† NdfR calculated from atom% ^{15}N excess values of roots and soils and soil N content (mg plant^{-1})

‡ Same letters following means ± standard errors (n=9) indicate no significant difference among treatments within each plant or soil component (P<0.05) according to Tukey's HSD test

§ BS, bulk soil; RS, rhizosphere soil

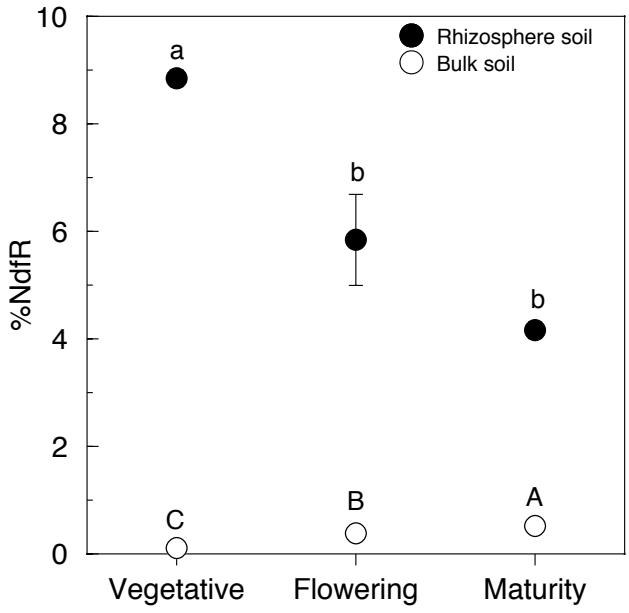

Fig. 4.3. Nitrogen-derived from rhizodeposition, NdfR (%), in the bulk and rhizosphere soils of pea grown in a greenhouse. Plants were supplied with ^{15}N-urea using cotton-wick ^{15}N labeling and were harvested at the early vegetative stage (32 DAS), flowering (55 DAS), and maturity (96 DAS). NdfR was calculated using the ^{15}N enrichment values of the roots and soil. Values are means ± standard errors (n=9). Within soil fractions, mean values labeled with the same lower- or uppercase letters indicate no significant difference in NdfR (%) between growth stages according to Tukey's HSD test (*P*>0.05).

4.6. Discussion

4.6.1. Recovery and distribution of ^{15}N in the plant-soil system

Recovery of added ^{15}N ranged from 75.5 to 88.9% for pea harvested at three different growth stages. These recovery rates are within the range of values reported in the literature for mature pea labeled using the cotton-wick method under greenhouse and field conditions (Mayer et al., 2003b; Wichern et al., 2007a). Rates of ^{15}N recovery were lowest for pea harvested at the vegetative stage, with only 75.5% of added ^{15}N recovered in the plant and soil components. These plants were harvested approximately one week after the last dose of ^{15}N was applied, while plants at flowering and maturity were harvested two and three weeks after the last dose of ^{15}N was applied, respectively. Therefore, there was less time for the ^{15}N-urea to absorb completely into the plants harvested during the earliest growth stage, despite the addition of deionized water to improve ^{15}N uptake following the final ^{15}N dose. Wichern et al. (2007b)

reported that 9% of added ^{15}N from urea was retained in the wick-labeling system for pea plants that received a single dose of ^{15}N one week prior to harvest at an early vegetative stage. Moreover, retention of added ^{15}N in the wick system was lower (2%) for plants that were harvested three to four weeks after application of ^{15}N-urea ceased (Wichern et al., 2007b). Therefore, the differences in percent ^{15}N recovery among the plant growth stages observed in the current study are probably due to greater retention of ^{15}N in the wick system at the earliest harvest stage.

Overall, the percentage of total recovered ^{15}N in soil remained relatively constant over the duration of ^{15}N labeling and plant growth, ranging from 5.8 to 5.0% between the vegetative growth stage and maturity. Under continuous ^{15}N labeling of nodulated pea using the cotton-wick method, Mahieu et al. (2009a) reported that only 2.0 and 1.7% of recovered ^{15}N was in soil at pod filling and maturity, respectively. In contrast, Wichern et al. (2007a) reported that 12.6 and 15.1% of the total ^{15}N recovered in pea was distributed in the soil at pod fill and maturity, respectively. The lower percentage of recovered ^{15}N distributed in soil in the present study and in Mahieu et al. (2009a) compared to that reported by Wichern (2007a) may be due to differences in ^{15}N labeling frequency and duration, as well as to the fact that the latter study was conducted in large contained soil cores buried in a field where rooting depth was not as restricted and where foliar growth was not as favored as in greenhouse pot studies.

Preferential ^{15}N enrichment of aboveground plant parts is commonly reported in studies that supply ^{15}N solutions directly to the plant stem (Mayer et al., 2003b; Russell and Fillery, 1996b; Wichern et al., 2007a; Wichern et al., 2010; Wichern et al., 2011). Consistent with these findings, we noted ^{15}N distribution ratios in aboveground plant components ranging from 1.01 to 1.12 and between 0.20 and 0.73 in roots and 0.40 and 0.70 in nodules, over all growth stages. The ^{15}N distribution ratios became closer to unity as ^{15}N labeling proceeded from the vegetative stage (0.25) to flowering (0.44) and maturity (0.66). Similar root ^{15}N distribution ratios in pea at pod filling and maturity were reported by Mahieu et al. (2009a). Likewise, Russell and Fillery (1996b) reported an increase in the ^{15}N distribution ratio of lupin roots with increased application of ^{15}N-urea, supporting a case for multiple rather than single additions of ^{15}N.

Nodules of actively fixing legumes tend to be less enriched in ^{15}N than their corresponding roots due to dilution with ^{14}N during N_2 fixation (Khan et al., 2002b; Russell and Fillery, 1996b). Uneven ^{15}N enrichment of nodulated roots potentially can result in overestimation of N

rhizodeposition if the ^{15}N enrichment of the recovered roots is lower than that of the rhizodeposits (Khan et al., 2002b). Atom% ^{15}N excess values and ^{15}N distribution ratios were greater in the nodules than the roots at the vegetative stage and flowering, indicating that the ^{15}N transferred from the aboveground components (i.e., originating from the ^{15}N supplied to the stem) to the roots and finally the nodules exceeded the amount of N derived though N_2 fixation. In fact, the higher ^{15}N enrichment of nodules compared to roots at the vegetative and flowering stages suggests that nodules were a stronger sink for ^{15}N than the roots. Conversely, uptake of ^{14}N from the soil during this early stage—when N fixation was minimal—may have diluted the ^{15}N in roots to a greater degree than in nodules, or was a consequence of accumulation of ^{15}N-urea in the nodules corresponding with carbohydrate transfer to the nodules. However, by the time pea reached maturity, ^{15}N enrichment and distribution in the nodules was lower than in the roots, suggesting that assimilation of ^{14}N during N_2 fixation had increased between early flowering and maturity. Jensen (1987) found that N_2 fixation was just over 5 kg N ha^{-1} during the flowering phase of pea, but peaked at over 10 kg N ha^{-1} at the onset of pod filling. Therefore, any contribution of symbiotically fixed N to soil probably was minimal, at least until early flowering and would have increased by maturity.

4.6.2. Plant N balance

Release of N from the roots of growing pea to the soil accounted for 17.9, 12.3, and 7.6% of total plant N assimilated at the vegetative stage, flowering, and maturity, respectively. Others who used the cotton-wick labeling method (Mahieu et al., 2009a; Mayer et al., 2003b; Wichern et al., 2007a; Wichern et al., 2007b), reported that N rhizodeposition by pea accounted for between 2.4 and 36.4% of total plant N at maturity. Wichern et al. (2007b) reported that NdfR accounted for 71.1% of total N at the early vegetative stage of pea, while in the present study NdfR comprised 17.9% of total N over roughly the same time period. Certainly, the large range in values reported in the literature reflects differences in the frequency and duration of ^{15}N-labeling—even when using the same ^{15}N labeling technique (in this case, the cotton-wick technique). For example, if the duration of ^{15}N labeling closely matches the growth period under study, the dilution of ^{15}N with newly assimilated ^{14}N in a growing root system can be reduced. This is important because any dilution of ^{15}N in the roots can lead to an overestimation of %NdfR. In an attempt to reduce this, ^{15}N was supplied to peas in frequent small doses

synchronized with N uptake (as determined in a preliminary experiment). Indeed, Mahieu et al. (2009a) found that estimates of NdfR were more accurate when pea was labeled with a continuous supply of ^{15}N rather than in fortnightly pulses.

Nitrogen fixation estimates for pea range from 0 to nearly 90%, with a median value of 55% for 79 studies conducted in the northern Great Plains (Walley et al., 2007). Applying the median value for N_2 fixation reported by Walley et al. (2007), the value for mean root N recovery (16 kg N ha^{-1}) in the field study by Gan et al. (2010b) and estimates of NdfR from the current study (i.e., 67.0% of total BGN at maturity), the total input of fixed N to soil was estimated as 26.7 kg N ha^{-1} for mature pea. A net deficit of N was likely to occur in this system if N fixation was 55% because the N harvest index (NHI = 0.93) for pea was relatively high. That is, the estimated amount of fixed N returned to the soil as crop residues, including aboveground residues, roots, and rhizodeposits (38.0 mg $plant^{-1}$ or 55% of total residue N) is less than the amount of soil-derived N exported in seed (144.6 mg $plant^{-1}$ or 45% of seed N). However, without the inclusion of N rhizodeposition in the total plant N balance the N deficit is overestimated by 15%. Of course, combining estimates from these field studies with the estimates derived from this greenhouse study may not reflect real conditions. In fact, NdfR may have been greater if estimates were made under field conditions where root to shoot ratios are likely to be higher (Wichern et al., 2008). Therefore, the estimates of fixed N in the root-derived N fraction may be conservative. Furthermore, in cases where NHI is lower than that reported here—and which is likely the case in the field (Gan et al., 2010b; Knight, 2012)—including root N and root-derived N may tip the balance in determining whether there is a net increase of fixed N to soil.

4.6.3. Temporal dynamics of N rhizodeposition

Nitrogen rhizodeposition comprised a higher proportion of plant N at the vegetative growth stage compared to subsequent growth stages. During this time root growth was expected to be rapid and root death minimal (Gavito et al., 2001). Therefore, the contribution of root-derived N to soil was most likely a result of N exudation from living roots. The relatively high atom% ^{15}N excess and %NdfR values in the rhizosphere soil relative to the bulk soil further support this hypothesis. Similarly, Ofosu-Budu et al. (1990) found that N release from soybean was greatest during the day rather than at night during the vegetative and flowering stages, while it was

greatest at night during pod filling. This suggests that active N exudation, rather than the release of N through root decomposition, was likely occurring at the two earliest growth stages relative to the pod filling stage. The authors suggest that N exudation was influenced by changes in membrane permeability or by changes in the rates of enzymatic processes involved in the degradation or synthesis of photosynthates (Ofosu-Budu et al., 1990). Rovira (1956) showed that soluble root exudates, rather than cell root debris, comprised a greater proportion of root material in 10-day-old compared to 20-day-old pea plants. Based on $^{14}CO_2$ labeling, root exudates—including sugars, carboxylic acids, and amino acids—decreased with plant age in corn under controlled conditions (Gransee and Wittenmayer, 2000). Plant age influences the partitioning of photoassimilate to the rhizosphere, with greater release of soluble exudates during early growth stages (Jones et al., 2009).

Root biomass (g plant^{-1}) decreased by 47% between flowering and maturity. Using mini-rhizotrons in a pot study, root growth of pea decreased at flowering, which also coincided with the onset of rapid root loss (Gavito et al., 2001). These observations are supported by data from the field; e.g., averaged over two growing seasons, Gan et al. (2009c) reported a decrease in root biomass and N of 30 and 36%, respectively, between late flowering (peak root biomass) and maturity. Using ^{15}N stable isotope labeling, we were able to account for some of the N loss to soil associated with the decline in root growth and the onset of root decomposition. For example, root-derived N in the bulk soil increased between flowering and maturity, from 22.0 to 29.0 mg N pot^{-1}, which was also associated with an increase in bulk soil ^{15}N. During this time, root atom% ^{15}N excess values increased by 23%; at the same time, atom% ^{15}N excess values of rhizosphere soil decreased by 14% while in the bulk soil they increased by 60%. The small relative change in atom% ^{15}N excess in the bulk soil resulted in an increase in %NdfR (from 0.40 to 0.52%). The change in %NdfR over time in the bulk soil suggests that the root-derived N was not likely a result of N exuded from living plant roots. Rather, the increased NdfR in the bulk soil probably more strongly represents the release of N from decomposition of roots that died between flowering and maturity. However, because of drier soil conditions at maturity there was proportionally less rhizosphere soil, and consequently more bulk soil, recovered at maturity compared to flowering, indicating that some of the elevated ^{15}N and NdfR in the bulk soil was due to lower recovery of rhizosphere soil. Therefore, rhizosphere soil sampling can also influence estimates of NdfR. Nevertheless, in a rhizobox study, detection of N rhizodeposits

extended only to 3–4 mm from the surface of a living wheat root (Schenck zu Schweinsberg-Mickan et al., 2012), providing further evidence that N rhizodeposition in bulk soil is likely a result of root turnover.

Distribution of recovered ^{15}N in the bulk soil remained relatively constant among all growth stages (ranging between 3.9 and 4.9% of total plant N), while distribution of total plant N in the bulk soil NdfR decreased over time (from 11.9 to 7.4%). This imbalance between total plant and soil ^{15}N and total plant N distribution in the bulk soil, particularly at the vegetative stage, may be due to the high ^{15}N distribution ratio in the aboveground components of pea during this stage. Mobilization of ^{15}N from aboveground plant components to roots may not have been favored during vegetative growth when the majority of N is supplied to the leaves (Schiltz et al., 2005). However, as pea developed from flowering through to pod filling and maturity, seeds became a strong sink for N and redistribution from vegetative components and roots likely occurred (Pate and Flinn, 1973; Schiltz et al., 2005). For example, 60% of ^{15}N was found in pea leaves at early stages of growth, while 70% of ^{15}N in the plant was remobilized towards seed at maturity (Atta et al., 2004). Root biomass decreased between flowering and maturity and total root N remained relatively stable during this time period. Therefore, while aboveground components continued to be a strong sink for ^{15}N between flowering and maturity, total ^{14}N assimilation increased and was further redistributed to aboveground components rather than to roots. As a result, a higher proportion of N was distributed aboveground, and particularly in seed at pea maturity, which may partially explain why the ^{15}N distribution ratio approached unity in the aboveground components as the plants matured. Not taking the aboveground N and ^{15}N distribution into account, distribution of soil ^{15}N and NdfR among the total belowground N was consistent across the growth stages.

4.7. Conclusion

Nitrogen rhizodeposition comprised an important part of the total plant N balance over the course of pea growth, ranging from 17.9% during early crop growth to 7.6% at maturity. However, the total input of N rhizodeposition to soil increased with plant development. Determining N rhizodeposition in both bulk and rhizosphere soils revealed that the predominant mechanism for N release from roots may change over time. Whereas N associated with root exudates from intact plant roots likely formed the dominant mode of N released from roots at the

vegetative stage, N released from root decay likely comprised a higher proportion of N rhizodeposition at crop maturity. However, further research involving qualitative assessment of root-associated compounds will be required to determine this with certainty. Providing a constant supply of ^{15}N using the cotton-wick technique allowed for the cumulative quantification of root-derived N over plant growth—an important factor in improving the estimation of the N budget of pulse crops.

5. ESTIMATING BELOWGROUND NITROGEN INPUTS OF PEA AND CANOLA AND THEIR CONTRIBUTION TO SOIL INORGANIC N POOLS USING ^{15}N LABELING[1]

5.1. Preface

In addition to assessing belowground crop residues to gain a more complete N budget for pulse crops, quantifying the input of N from belowground residues of non-legumes is an important starting point in determining the fate of crop residue N in agricultural soils. Chapter 4 quantified N rhizodeposition in field pea; in this Chapter, assessments of N rhizodeposition in pea will be compared with that of canola, the major oilseed crop grown in Canada. The goal of this study was to compare the total contribution of belowground inputs of N to the total crop residue N budget, as well as to determine the partitioning of N rhizodeposition in soil inorganic N pools to evaluate differences in the quality of the rhizodeposits between the two crop species.

5.2. Abstract

Crop species grown in a diversified crop rotation can influence soil N dynamics to varying degrees due to differences in the quantity and quality of the residues returned to the soil. The aim of this study was to quantify the contribution of N rhizodeposition by canola (*Brassica napus* L.) and pea (*Pisum sativum* L.) to the crop residue N balance and soil inorganic N pool. Canola and pea were grown in a soil-sand mixture and were subject to cotton-wick ^{15}N labeling in a greenhouse experiment. Nitrogen-15 recovered in the soil and roots were used to estimate N rhizodeposition. Belowground N, including root N and N rhizodeposits, comprised 70% and 61% of total crop residue N for canola and pea, respectively. Canola released the greatest amount of total root-derived N to the soil, which was related to greater root biomass production by canola. However, root-derived N in the soil inorganic N pool was greater under pea (13%) than

[1]Chapter 5 of this dissertation has been previously published (with minor changes for formatting) as: Arcand, M.M., J.D. Knight, and R.E. Farrell. 2013. Estimating belowground nitrogen inputs of pea and canola and their contribution to soil inorganic N pools using ^{15}N labeling. Plant Soil:1-14. I designed and implemented the experiment, collected and analyzed samples, as well as conducted statistical analyses, interpreted the results, and was the primary author of the manuscript.

canola (4%). Our results show a significant belowground N contribution to total crop residue from pea and canola. Further investigation is required to determine whether input of the more labile N rhizodeposits of pea improves soil N supply to succeeding crops or increases the potential for N loss from the soil system relative to canola.

5.3. Introduction

A diverse crop rotation is an integral component of a sustainable agricultural system. Increasing the diversity of crops grown within a rotation can enhance nutrient and water use efficiency (Tilman et al., 2002), suppress plant disease and weed pressure (Cardina et al., 2002; Krupinsky et al., 2002), reduce reliance on exogenous inputs (Hanson et al., 2007), and decrease the carbon footprint (Gan et al., 2011). In the northern Great Plains of North America, producers have diversified and extended their crop rotations to include pulse and oilseed crops into what historically was a cereal-fallow system (Liebig et al., 2007). Pulse crops are annual legume crops produced for edible seed. While wheat continues to be the dominant crop grown in the Canadian prairies, occupying 8.6 million ha of land annually, it is now most likely to be grown in rotation with canola and pea, which represent the dominant oilseed and pulse crops grown in this region, respectively (Statistics Canada, 2011). In these cropping systems, animal manure inputs are limited and plant inputs via return of crop residues and rhizodeposits are the primary sources of organic material, providing energy and nutrients for the soil microorganisms that drive nutrient cycling and build soil organic matter (Soon and Arshad, 2002). Moreover, differences in the quantity and quality of the crop residue input to the soil will vary among crop species, and may influence plant N availability both within and following the growing season.

Amounts of soil inorganic N have been reported to be higher at harvest of mature field pea and canola relative to cereal crops (Engström and Lindén, 2012; Kirkegaard et al., 1999; Ryan et al., 2006). In some cases, accumulation of soil inorganic N also was reported to be higher following canola compared to pea (Kirkegaard et al., 1999; Ryan et al., 2006), possibly due to nitrification inhibition by-products released during the hydrolysis of glucosinolate contained in canola residues (Brown and Morra, 2009). Conversely, Gan et al. (2010b) reported that amounts of soil inorganic N at harvest were higher under wheat than either canola or pea, but that N mineralization over the growing season was nearly twice as great under pea and wheat compared to canola. In general, pea and canola return higher amounts of N to the soil than cereals due to

higher concentrations of N in the root and aboveground residues (Gan et al., 2010b; Soon and Arshad, 2002). The release of N to soil from roots within the growing season may further explain differences in inorganic N observed among crop species at harvest. A proportion of labile root-derived organic N compounds can be mineralized (Janzen, 1990), and/or inorganic N can be released directly from roots (Brophy and Heichel, 1989). Nitrogen released to the soil through rhizodeposition contributed 25 to 46% of inorganic N from mature pulse crops (pea, faba bean, and white lupine) and oats (Mayer et al., 2003b; Wichern et al., 2007b).

Nitrogen rhizodeposition in pea has been studied extensively compared to other field crops (Fustec et al., 2010; Wichern et al., 2008). In a review of N rhizodeposition, Wichern et al. (2008) report that the median distribution of pea N rhizodeposition as a proportion of total plant N and total belowground N was 13% and 79%, respectively, across field and greenhouse studies. While there has been much focus on quantifying N rhizodeposition in pulse crops due to their ability to biologically fix N (Fustec et al., 2010; Wichern et al., 2008), the few studies that have been conducted on non-N fixing field crops have focused on cereals (De Graaff et al., 2007; Janzen and Bruinsma, 1989; Jensen, 1996c; Wichern et al., 2007a; Wichern et al., 2007b). Among thirteen studies reviewed, the median distribution of wheat N rhizodeposition as a proportion of total plant N and belowground N was 13% and 67%, respectively (Wichern et al., 2008). Recently, shoot ^{15}N-labeling techniques have been used to quantify total N rhizodeposition, and its contribution to soil inorganic N (Mayer et al., 2003b; Wichern et al., 2007a; Wichern et al., 2007b). To date, no estimates of N rhizodeposition in canola have been reported; there is no information on the influence of N rhizodeposition on amounts of soil inorganic N following canola or on the total contribution of N rhizodeposition to the total plant N balance. In order to evaluate the contribution of different crops to N cycling and optimize their role in N-efficient cropping sequences it is critical that accurate estimates of belowground contributions be developed.

The purpose of this study was to estimate N rhizodeposition from canola and pea. The cotton-wick ^{15}N labeling technique was used to quantify N rhizodeposition as it can supply frequent doses of ^{15}N solution to the plant throughout growth (Mahieu et al., 2009a), thus providing a more homogenous distribution of ^{15}N within the plant compared to other shoot-labeling methods (Mayer et al., 2003b). Our specific objectives were to determine the partitioning of ^{15}N within the plant and soil, including inorganic N pools, and to determine the

contribution of N rhizodeposition to total plant N and total crop residue N (straw, roots, and root-derived N in soil) for canola and pea in a greenhouse study. Nitrogen fertilized canola and non-fertilized, inoculated, pea were compared since these treatments represent common agronomic practice. An additional N-fertilized pea treatment was included to evaluate any differences specifically due to fertilization. Nitrogen rhizodeposition was defined as the root-derived N remaining in the soil after removal of visible roots and root fragments.

5.4. Materials and Methods

5.4.1. Experimental setup

Soil—classified as an Orthic Brown Chernozem (Ayers et al., 1985)—was collected from Agriculture and Agri-Food Canada's Semiarid Prairie Agricultural Research Centre (SPARC) located at Swift Current, Saskatchewan, Canada (50°15'N, 107°44'W). Following collection, the soil was air dried, sieved (4 mm) to remove any rocks, and mixed with silica sand in a 1:1 (w/w) ratio. The resulting soil-sand medium was loam in texture, had a pH of 7.1 (1:2 soil:H_2O), and contained 0.06% total N, 9 mg NO_3^--N kg^{-1}, 29 mg P kg^{-1}, 220 mg K kg^{-1}, and 3.6 mg S kg^{-1}. Soils were packed to a bulk density of 1.4 g cm^{-3} in pots (20 cm dia., 20 cm deep) that were lined with a plastic bag to prevent solution loss via leaching. Five pea (cv. CDC Meadow) seeds or five canola (cv. 45H21) seeds were planted per pot on July 26, 2010 and thinned to one plant per pot following germination. At the time of seeding, liquid inoculant containing *Rhizobium leguminosarum* bv. *viciae* (Nodulator®XL, Becker Underwood, Saskatoon, SK) was applied to one-half of the pea seed according to the manufacturer's instructions at the recommended rate equivalent to 75 mL per 27.3 kg of seed. Plants were watered every other day with deionized water to achieve approximately 80% field capacity. All plants were supplied with 32 mg P pot^{-1} (20 kg P ha^{-1}) as KH_2PO_4. The pea plants that were not inoculated and all of the canola plants received 192 mg N pot^{-1} (120 kg N ha^{-1}) as urea. The N-fertilized pea was not inoculated so that it would rely on fertilizer- and soil-N to meet its N requirement, as was the case for canola. The pots were arranged on a greenhouse bench as a randomized complete block design with eight replicate pots for both the ^{15}N labeled plants (3 crop treatments × 8 replicates) and the natural abundance control plants (3 crop treatments × 8 replicates) for a total of 48 pots.

5.4.2. ^{15}N Labeling Method

The method for introducing ^{15}N-enriched urea into the plants was based on the cotton-wick method first described by Russell and Fillery (1996b), and modified by Mayer et al. (2003b) and Mahieu et al. (2009a). Briefly, a 0.5 mm hole was drilled into the stem of the plant, approximately 5 cm above the soil surface. A cotton thread was fed through the hole in the stem using a thin needle—with both ends of the cotton wick threaded through silicone tubing (0.76 mm i.d. × 4 cm length) to reduce evaporative losses. One end of the silicone tubing was adhered to the stem of the plant using plasticine; the other end, and the enclosed cotton thread, were fed through the cap of a 2 mL vial and the thread immersed in a 0.3% (w/v) ^{15}N enriched urea solution (99.2 atom% ^{15}N). The cap of the vial consisted of a Teflon coated silicone septum with a hole small enough to allow for the silicone tubing and the thread to feed through, but provided sufficient seal to minimize solution evaporation. A needle and syringe were used to replenish the ^{15}N-urea solution over the course of the experiment in small doses (0.20–0.35 mL) through the septum of the cap. The frequency of ^{15}N-urea application depended on the rate of solution uptake: in the first three weeks of labeling, urea was applied every 2 to 4 d; in the last two weeks, applications were spaced at 5 to 6 d intervals due to slower solution uptake. As the labeling proceeded, the plant stems developed scar tissue surrounding the cotton wick that prevented efficient solution uptake. As a result, reinstallation of the wick system was required on at least one occasion for all plants, and two or three times for a few plants. Following plant uptake of the last addition of ^{15}N urea solution, 0.40 mL of deionized water was added to maximize ^{15}N urea solution uptake from the wick into the plant stem. Pea and canola plants were supplied with 3.9 and 3.8 mg ^{15}N plant^{-1}, respectively, as urea from 21 to 56 days after sowing (DAS), which corresponded to late flowering. Labeling began at the 6–7 leaf stage for pea and 5–6 leaf stage for canola.

5.4.3. Soil and plant sample preparation and analysis

Pea was harvested at 100 DAS and canola at 130 DAS, with the aboveground plant components separated into leaves and stems, pods, and seed, and dried at 60°C. The pots containing soil and the intact roots were stored at 2°C until roots could be removed from the soil. The canola plants shed leaves throughout the experiment, as is commonly observed (Malagoli et al., 2005); the shed leaves were collected and later processed and analyzed for ^{15}N at the same

time as the plant parts harvested at maturity. The pea plants did not shed leaves. Roots and visible root fragments were removed from the soil using a 2 mm sieve and tweezers; soil from which roots were removed was considered bulk soil. The soil adhering to the roots was considered rhizosphere soil. Roots were washed over a 0.5 mm sieve with deionized water, dried at 60°C, and finely ground in a freezer mill. All of the soil-water slurry was collected during root washing and any visible root fragments that passed through the sieve were removed with tweezers. The soil-water slurry was dried at 75°C to recover the rhizosphere soil. All aboveground plant samples and soils were finely ground in a ball mill prior to analysis. Whereas there were no nodules on six of the eight non-inoculated, N-fertilized pea plants, a few nodules (<5) were present on two plants. Nodules were observed on all of the roots of the non-fertilized, inoculated pea. Pea roots and nodules were processed together.

Subsamples of the rhizosphere and bulk soil were collected immediately following aboveground sample collection and prior to complete root sampling. This was done to expedite the sampling and storage of soil that would be used for inorganic N analysis. Following gentle shaking of the root system, rhizosphere soil subsamples (1.5 and 1.0 g for canola and pea, respectively) were removed from sections along the primary and lateral roots using tweezers and stored in 1.5 mL Eppendorf tubes. At the same time roots also were removed from a subsample of soil and this bulk soil (80 g) stored in 50 mL Falcon tubes. The subsamples of the rhizosphere and bulk soils were stored at -20°C. The frozen bulk and rhizosphere soil subsamples were thawed and combined in proportions equal to the proportions of total rhizosphere to total bulk soil (i.e., subsampled soils plus soils recovered after root sampling) for inorganic N and ^{15}N analysis. Soil NO_3^--N and NH_4^+-N from these thawed composite soil samples were extracted with 2 *M* KCl and quantified colorimetrically using a SmartChem™ 200 (Westco Scientific Instruments, Brookfield, CT). Enrichment of ^{15}N in both the NO_3^- and NH_4^+ pools in the 2.0 *M* KCl extracts were determined by analyzing the ^{15}N diffused onto acidified diffusion disks encased in polytetrafluoroethylene tape according to the method of Stark and Hart (1996).

Diffusion disks and finely ground soil and plant samples were weighed into tin capsules and analyzed for N concentration (%) and $\delta^{15}N$ using an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific Inc., Waltham, MA) coupled to an elemental analyzer (Costech ECS4010, Costech Analytical Technologies, Inc., Valencia, CA). The atom% ^{15}N values of the soils and plants grown under natural abundance conditions were subtracted from

the atom% ^{15}N values of soil and plant components of ^{15}N-labeled plants to yield atom% ^{15}N excess values for each soil and plant component. The natural abundance atom % ^{15}N values of the soil $^{15}NO_3^-$ (0.3717 atom% ^{15}N) and $^{15}NH_4^+$ (0.3737 atom% ^{15}N) pools used to determine the atom% ^{15}N excess values of those pools were calculated from the natural abundance of total soil (0.3690 atom% ^{15}N) using a correction factor of 0.73% (NO_3^-) and 1.27% (NH_4^+) obtained from the percent difference in the natural abundance values of the total soil and soil NO_3^- and NH_4^+ pools determined in a companion study of the same soil.

5.4.4. Calculations

The percentage of soil N derived from rhizodeposition (% NdfR) was calculated based on the assumption that the ^{15}N enrichment of the recovered (visible) roots was the same as the ^{15}N enrichment of the root-derived N deposited into the soil, according to the equation (Janzen and Bruinsma, 1989):

$$\%\text{NdfR} = \frac{\text{atom\% } ^{15}\text{N soil} - \text{atom\% } ^{15}\text{N background A}}{\text{atom\% } ^{15}\text{N roots} - \text{atom\% } ^{15}\text{N background B}} \times 100 \qquad [5.1]$$

where background-A is the atom% ^{15}N in the soil in which non-labeled plants were grown and background-B is the atom% ^{15}N in the roots of non-labeled plants (Schmidtke, 2005b). Quantities of NdfR were calculated by multiplying %NdfR by the total N (mg pot^{-1}) in the particular soil pool of interest. These calculations represent net N rhizodeposition; i.e., they do not consider reabsorption of N by the plant. Use of the Janzen and Bruinsma equation also assumed uniform distribution of ^{15}N within the root system and constant ^{15}N enrichment over the plant growth period (Mayer et al., 2003b).

Nitrogen-15 distribution ratios were calculated as the amount of ^{15}N excess in a plant component divided by the total amount of ^{15}N excess in all plant components (i.e., % of total ^{15}N recovered) and the amount of N in the same plant component divided by the total amount of N in all plant components (% of total plant N) (Russell and Fillery, 1996b). Soil ^{15}N and estimates of NdfR were not included in the calculation. A value of one indicates uniform ^{15}N distribution, values greater than one indicate preferential ^{15}N enrichment, and values less than one indicate discrimination against ^{15}N enrichment within a particular plant component (Russell and Fillery, 1996b). These values offer a more meaningful indication of ^{15}N distribution than atom% ^{15}N excess values alone since the latter are confounded by differences in total plant N, ^{15}N uptake

from the solution, and N uptake from fertilizer, soil, and atmospheric sources (Russell and Fillery, 1996b).

5.4.5. Statistical analysis

Statistical analyses were performed using SPSS® Statistics version 20.0 for Mac (IBM Corp., 2011). One-way analysis of variance was performed with crop species as a fixed effect and block as a random effect. Comparison of atom% ^{15}N excess values between the ^{15}N-labeled treatments and the ^{15}N-natural abundance controls within a plant or soil component were completed using a two-tailed Student's t-test. Normality of residuals was tested using the Shapiro-Wilk statistic and homogeneity of variances was tested using Levene's test. Means comparisons were made using Tukey's Honestly Significant Differences. When the assumptions of the ANOVA were not met, data were analyzed using Kruskal-Wallis; if this test was significant, pair-wise comparisons were made between treatments using the Mann-Whitney test. All tests were declared significant at $P<0.05$.

5.5. Results

5.5.1. Plant biomass and distribution

Total biomass, on a per plant basis, was greater for canola than for pea (Table 5.1). Nitrogen fertilization significantly increased total pea biomass by 38%, with the largest increase (55%) occurring in the seed biomass. Root and foliar biomass were not enhanced by N fertilization.

Given the different growth characteristics of pea and canola, it is more meaningful to compare the distribution of plant biomass rather than the absolute biomass between the two crop species. A higher proportion of total plant biomass was allocated towards seed development in pea compared to canola (Table 5.1). Relative to pea, a higher proportion of the total biomass in canola was allocated to foliar growth (51%) and root development (19%). Fertilizing pea with N increased biomass partitioning to seed by 12.7% compared to non-fertilized, inoculated, pea but had no affect on biomass partitioning to other plant parts (Table 5.1).

Table 5.1. Dry biomass (g plant^{-1}) and biomass distribution (%) of plant components of mature canola, N-fertilized pea, and non-fertilized pea grown in a greenhouse and supplied with ^{15}N-urea using the cotton-wick labeling method.

Crop treatment	Seed	Pods	Leaves and stems	Roots	Total
			Biomass (g plant^{-1})		
Canola	2.59 ± 0.18 c†	2.79± 0.19 a	9.50 ± 0.93 a	3.38 ± 0.09 a	18.26 ± 0.81 a
Pea N-fertilized	5.45 ± 0.17 a	1.07 ± 0.07 b	3.55 ± 0.31 b	0.40 ± 0.06 b	10.46 ± 0.48 b
Pea non-fertilized	3.51 ± 0.21 b	0.86 ± 0.16 b	2.90 ± 0.23 b	0.32 ± 0.02 b	7.59 ± 0.49 c
P value	<0.001	<0.001	<0.001	<0.001	<0.001
			Biomass distribution (%)		
Canola	14.5 ± 1.4 c	15.7 ± 1.6 a	51.1 ± 3.5 a	18.7 ± 0.8 a	100
Pea N-fertilized	52.5 ± 1.9 a	10.2 ± 0.3 b	33.5 ± 1.7 b	3.8 ± 0.5 b	100
Pea non-fertilized	46.6 ± 4.5 b	11.1 ± 4.4 b	38.0 ± 1.4 b	4.2 ± 0.7 b	100
P value	<0.001	0.010	<0.001	<0.001	

† Same letters following means ± standard errors (n=8) indicate no significant difference among treatments within each plant part (P>0.05) according to Tukey's HSD test

5.5.2. Atom% ^{15}N excess in plant components and bulk and rhizosphere soils

Non-fertilized pea had higher atom% ^{15}N excess values in aboveground plant parts than canola (Table 5.2). When pea was fertilized with urea, atom% ^{15}N excess in aboveground plant parts was more similar to the fertilized canola. Indeed, seed and pod atom% ^{15}N excess were the same for fertilized canola and pea (P>0.05) and atom% ^{15}N excess in leaves and stems of the fertilized pea was intermediate between canola and non-fertilized pea. Root enrichment did not differ among crop treatments, but was lower relative to atom% ^{15}N in aboveground plant parts.

Soil enrichment was markedly lower than plant ^{15}N enrichment (Table 5.2). Despite this, ^{15}N labeling resulted in significant ^{15}N enrichment in both rhizosphere and bulk soils compared to the ^{15}N levels in the natural abundance control soils (P<0.001). Moreover, averaged across all of the crop treatments ^{15}N enrichment of the rhizosphere soil (0.0535 atom% ^{15}N excess) was greater than that of the bulk soil (0.0159 atom% ^{15}N excess; P<0.001). Enrichment of rhizosphere soil followed the order: non-fertilized pea = canola ≥ N-fertilized pea. In contrast, enrichment of the bulk soil followed a different pattern: canola > non-fertilized pea = N-fertilized pea.

5.5.3. ^{15}N distribution ratios within the plant

Root ^{15}N distribution ratios were less than one for both crops, but were greater for canola (0.54 ± 0.04; P=0.023) than for pea (0.37 ± 0.04). The ^{15}N distribution ratio of all aboveground components (seed, leaf and stem, pod) was greater than one for both crops and significantly higher (P<0.001) for canola (1.07 ± 0.005) than for pea (1.03 ± 0.003), indicating that the aboveground components of canola were preferentially enriched in ^{15}N to a greater degree than they were for pea, regardless of the N fertility treatment in pea.

5.5.4. Recovery of ^{15}N and its distribution in plant and soil

Recovery of the added ^{15}N (i.e., supplied to the plants) in the soil-plant system ranged from 77 to 88% and tended to be greater in pea than in canola (P=0.051; data not shown). Total ^{15}N recovered in the N-fertilized and non-fertilized pea was 3.468 and 3.352 mg ^{15}N plant^{-1}, respectively, whereas in canola total ^{15}N recovery was 2.948 mg ^{15}N plant^{-1}.

Table 5.2. Atom % ^{15}N excess, recovery of added ^{15}N (%), and distribution of recovered ^{15}N (%) in plant components and soil of mature canola, N-fertilized pea, and non-fertilized pea grown in a greenhouse and supplied with ^{15}N-urea using the cotton-wick labeling method.

Crop treatment	Plant				Soil		Total
	Seed	Pods	Leaves and stems	Roots	Rhizosphere	Bulk	
				Atom% ^{15}N excess			
Canola	1.86 ± 0.18 b†	1.86 ± 0.09 b	1.90 ± 0.07 b	0.92 ± 0.03 a	0.0588 ± 0.0041 ab	0.0267 ± 0.0035 a	----
Pea N-fertilized	1.95 ± 0.07 b	1.59 ± 0.06 b	2.25 ± 0.26 ab	0.70 ± 0.07 a	0.0364 ± 0.0036 b	0.0093 ± 0.0001 b	----
Pea non-fertilized	2.89 ± 0.30 a	2.89 ± 0.16 a	2.94 ± 0.38 a	1.03 ± 0.13 a	0.0654 ± 0.0077 a	0.0118 ± 0.0010 b	----
P value	0.002	<0.001	0.032	0.081	0.012	<0.001	
				Distribution of recovered ^{15}N (%)			
Canola	53.2 ± 4.6 b	4.5 ± 0.4 a	18.1 ± 2.7 a	6.0 ± 0.4 a	0.45 ± 0.04 a	18.3 ± 2.7 a	100
Pea N-fertilized	78.9 ± 2.0 a	1.8 ± 0.1 b	12.3 ± 1.9 a	1.4 ± 0.2 b	0.10 ± 0.02 b	5.4 ± 0.3 b	100
Pea non-fertilized	75.0 ± 1.7 a	2.5 ± 0.5 b	13.8 ± 1.1 a	1.7 ± 0.2 b	0.16 ± 0.03 b	6.9 ± 0.7 b	100
P value	<0.001	0.001	0.166	<0.001	<0.001	<0.001	

† Same letters following means ± standard errors (n=8) indicate no significant difference among treatments within each plant or soil component (P>0.05) according to Tukey's HSD test

For each crop, the majority of the recovered ^{15}N was distributed in the harvested seed (Table 5.2). Moreover, whereas there was no difference between crops in the percentage distribution of ^{15}N in leaves and stems, there were clear differences in the allocation of ^{15}N to seed; i.e., 75 to 79% of recovered ^{15}N in pea was found in the seed, but only about 53% of recovered ^{15}N in canola was found in the seed. Distribution of recovered ^{15}N in soil was similar between N-fertilized and non-fertilized pea (5.4–6.9%), but was greater for canola, with 18.3% of ^{15}N recovered in the bulk soil. A similar pattern was observed for ^{15}N distribution in roots, with 6.0% found in the roots of canola, but only 1.4 to 1.7% found in the roots of pea. Of the total ^{15}N recovered in plant parts and soil, belowground ^{15}N (roots and soil) comprised 6.9, 8.8, and 24.8% in N-fertilized pea, non-fertilized pea, and canola, respectively (Table 5.2).

Relative to the total amount of ^{15}N in the crop residues remaining in the soil system following seed harvest, the aboveground residue (leaves, stems, and pods) contributed the highest proportion of recovered ^{15}N for pea (66%; Fig. 5.1a). In contrast, belowground residue (including intact roots and N rhizodeposition) contributed the highest proportion of recovered ^{15}N for canola (52%; Fig. 5.1a). For both pea and canola, root-derived ^{15}N in soil comprised a greater proportion of total residue ^{15}N than intact roots.

5.5.5. Total plant N and N rhizodeposition and the distribution of N in plant and soil

Canola and non-fertilized pea had comparable total amounts of N in the recoverable plant parts (i.e., excluding rhizodeposits; Table 5.3). However, including estimates of NdfR in the bulk and rhizosphere soils as part of total plant N significantly increased the total plant N accumulated in the canola system, resulting in higher amounts of N in canola than non-fertilized pea, whereas fertilizing pea resulted in parity with canola. Nitrogen in the canola system was approximately 40% greater than that in the non-fertilized pea system (Table 5.3). Total seed N was similar for canola and non-fertilized pea (Table 5.3). However, seed N represented only 43.2% of the total N in canola, and 64.5% of the total N in non-fertilized pea. Nitrogen fertilization of pea increased the amount of seed N by 50% (94.0 to 140.8 mg plant^{-1}). However, as a proportion of total plant N, there was no significant difference in N allocated to seed between the non-fertilized and N-fertilized pea (Table 5.3). The total amount of N allocated to roots was greatest in canola (Table 5.3), despite the fact that the N concentration of the canola roots was low (0.54%) relative to pea (1.78%, data not shown). There was a positive linear relationship between root biomass

and total NdfR (r^2=0.60, P<0.001).

Including estimates of NdfR in the total N budget of pea and canola shows that N rhizodeposition comprised a significant proportion of total plant N (Table 5.3). In canola, NdfR in the bulk soil totaled 58.2 mg N plant^{-1} (or 29% of the total plant-derived N). In the non-fertilized pea, NdfR in the bulk soil totaled 23.3 mg N plant^{-1} (14% of the total plant N). Fertilizing pea resulted in only a small increase in NdfR in the bulk soil compared to non-fertilized pea. In both canola and pea, however, NdfR in the rhizosphere accounted for only about 2% of the total NdfR, regardless of the fertilization status of the pea.

The amount of total crop residue N—including intact roots, NdfR, leaves, stems, and pods—remaining in the soil system following harvest comprised 57.0 and 35.6% of total plant N for canola, and non-fertilized pea, respectively (Table 5.3). Fertilizing pea did not increase the relative contribution of the total crop residue N to the soil system compared to non-fertilized pea. Only 29.2% of the total crop residue N of fertilized pea remained in the soil system following harvest. Root N alone comprised 16.4% and 11.5% and NdfR in soil 51.5% and 49.1% of total residue N for canola and non-fertilized pea, respectively (Fig. 5.1b). Consequently, total belowground contributions to crop residue N were greater than aboveground contributions from leaf, stem, and pod residue.

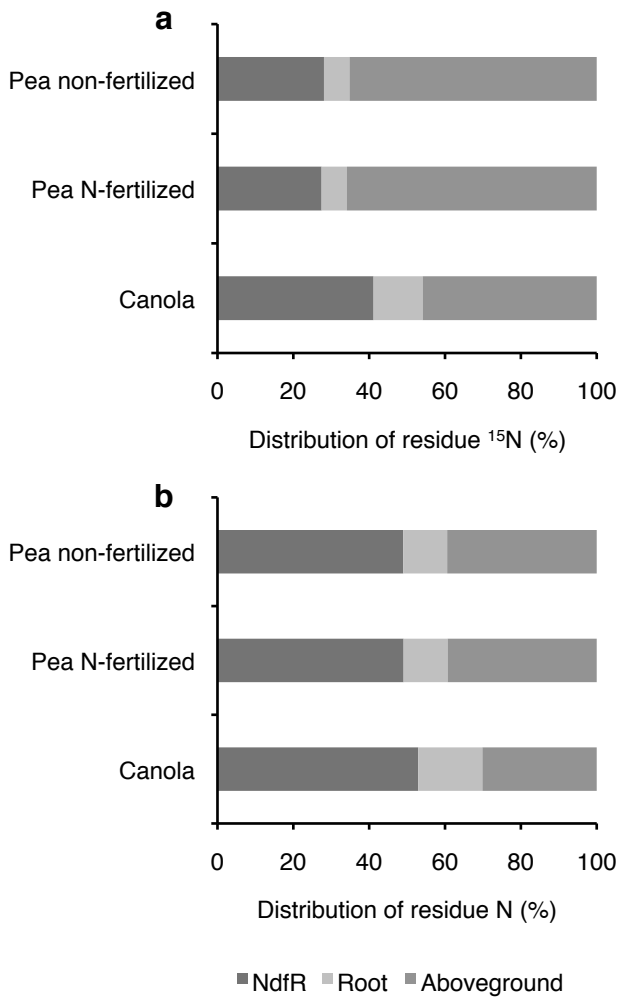

Fig. 5.1. Distribution of ^{15}N (a) and total plant N (b) in crop residues, including aboveground, root, and N derived from rhizodeposition (NdfR) in soil, of mature canola, N-fertilized pea, and non-fertilized pea grown in a greenhouse and supplied with ^{15}N-urea using the cotton-wick labeling method.

Table 5.3. Total N in each plant component (mg N plant^{-1}), N derived from rhizodeposition (NdfR) in soil (mg N pot^{-1}), and the distribution of N in each soil and plant component as a proportion of total plant N (%) for mature canola, N-fertilized pea, and non-fertilized pea grown in a greenhouse and supplied with ^{15}N-urea using the cotton-wick labeling method.

Crop Treatment	Plant				Soil		Total	
	Seed	Pods	Leaves and stems	Roots	$NdfR_{RS}$†	$NdfR_{BS}$	Plant only	Plant and NdfR
	mg N plant^{-1}				*mg N pot^{-1}*‡		*mg N pot^{-1}*	
Canola	84.7 ± 5.7 b§	7.4 ± 0.5 a	30.4 ± 4.1 a	17.9 ± 0.5 a	1.5 ± 0.2 a	58.2 ± 8.2 a	139.9 ± 4.9 b	199.5 ± 9.6 a
Pea N-fertilized	140.8 ± 3.0 a	4.0 ± 0.2 b	18.8 ± 1.5 b	6.8 ± 0.9 b	0.5 ± 0.1 b	28.3 ± 2.7 b	170.4 ± 1.8 a	199.2 ± 3.1 a
Pea non-fertilized	94.0 ± 9.1 b	2.9 ± 0.5 b	16.3 ± 1.4 b	5.5 ± 0.3 b	0.5 ± 0.1 b	23.3 ± 2.1 b	118.8 ± 10.6 b	142.5 ± 11.8 b
P value	<0.001	<0.001	0.006	<0.001	<0.001	<0.001	0.003	0.001
	Distribution of total plant N (%)							
Canola	43.2 ± 3.4 b	3.7 ± 0.4 a	14.8 ± 0.8 a	9.1 ± 0.4 a	0.75 ± 0.09 a	28.6 ± 2.8 a	70.8 ± 2.8 b	100
Pea N-fertilized	70.8 ± 1.6 a	2.0 ± 0.1 b	9.4 ± 0.8 a	3.4 ± 0.4 b	0.25 ± 0.04 b	14.1 ± 1.2 b	85.6 ± 1.2 a	100
Pea non-fertilized	64.5 ± 1.9 a	2.1 ± 0.4 b	11.9 ± 1.0 a	4.1 ± 0.2 b	0.36 ± 0.04 b	17.1 ± 1.4 b	82.6 ± 1.4 a	100
P value	<0.001	0.001	0.045	<0.001	<0.001	0.001	0.001	

† RS, rhizosphere soil; BS, bulk soil

‡ Quantities of NdfR in rhizosphere and bulk soils was determined by multiplying NdfR (%) calculated with the Janzen and Bruinsma equation (1989) and total N (mg pot^{-1})

§ Same letters following means ± standard errors (n=8) indicate no significant difference among treatments within each plant or soil component (P>0.05) according to Tukey's HSD test

5.5.6. Soil inorganic N and partitioning of ^{15}N in NH_4^+ and NO_3^- pools

Approximately 61% more NO_3^--N was recovered under pea than canola at harvest (Table 5.4). Concentrations of NH_4^+-N were 12- to 15-times lower than those of NO_3^--N, ranging from 1.28 to 2.84 mg pot^{-1}.

Although there was less NO_3^--N in soils grown under canola (Table 5.4), the higher atom% ^{15}N excess in this pool (data not shown) resulted in a total $^{15}NO_3^-$-N (μg pot^{-1}) content that was not statistically different from that of the non-fertilized pea (Table 5.4). Nevertheless, there was a trend suggesting that total $^{15}NO_3^-$-N was greatest for non-fertilized pea (P=0.064).

More total ^{15}N was recovered in soils (bulk + rhizosphere) under canola than under either pea treatment (551.96 μg ^{15}N pot^{-1} for canola *vs*. 181.25 and 235.64 μg ^{15}N pot^{-1}, for N-fertilized and inoculated pea, respectively). A larger amount of ^{15}N was recovered in the organic N pool under canola than under pea since the amount of ^{15}N was similar in the inorganic N pool for all crop treatments (Table 5.4). Similarly, while there was no difference in the amount of inorganic NdfR among crop treatments, organic NdfR was greater for canola than pea.

Nitrate-N comprised 4.0% and 12.8% of total NdfR in soils grown with canola, and non-fertilized pea, respectively. Fertilizing pea had no effect on the partitioning of N rhizodeposition in the soil inorganic N pool or on the accumulation of total soil inorganic N. There was no significant treatment effect for NH_4^+-N,-^{15}N, or -NdfR.

Table 5.4. Total N (mg N pot^{-1}), ^{15}N enrichment (μg ^{15}N pot^{-1}) and N derived from rhizodeposition (mg NdfR pot^{-1}) in soil N pools following growth and harvest of mature canola, N-fertilized pea, and non-fertilized pea grown in a greenhouse and supplied with ^{15}N-urea using the cotton-wick labeling method.

Crop treatment	NO_3^-	NH_4^+	Organic N pools†
		mg N pot^{-1}	
Canola	19.19 ± 0.78 b‡	1.28 ± 0.38 a	1965.49 ± 26.72 a
Pea N-fertilized	32.10 ± 1.80 a	2.84 ± 1.08 a	1992.73 ± 43.42 a
Pea non-fertilized	29.72 ± 0.89 a	2.03 ± 0.54 a	1954.81 ± 43.40 a
P value	<0.001	0.345	0.905
		μg ^{15}N pot^{-1}	
Canola	22.16 ± 2.05 a	0.34 ± 0.14 a	529.46 ± 72.78 a
Pea N-fertilized	23.36 ± 2.31 a	0.11 ± 0.05 a	163.78 ± 10.47 b
Pea non-fertilized	30.17 ± 2.78 a	0.41 ± 0.20 a	205.06 ± 23.80 b
P value	0.077	0.271	<0.001
		mg NdfR pot^{-1}§	
Canola	2.390 ± 0.200 a	0.038 ± 0.016 a	57.215 ± 8.003 a
Pea N-fertilized	3.489 ± 0.522 a	0.015 ± 0.007 a	23.513 ± 1.956 b
Pea non-fertilized	3.050 ± 0.266 a	0.049 ± 0.026 a	20.687 ± 2.066 b
P value	0.215	0.320	<0.001

† Calculated as the difference from total N and NO_3^--N and NH_4^+-N

‡ Same letters following means ± standard errors (n=8) indicate no significant difference among treatments within each soil pool (P>0.05) according to Tukey's HSD test

§ Quantities of NdfR in each soil N pool were determined by multiplying NdfR (%) calculated with the Janzen and Bruinsma equation (1989) and total N in the corresponding soil pool (mg pot^{-1})

5.6. Discussion

5.6.1. Plant biomass yield and partitioning

Despite a lower plant density, Gan et al. (2009a) found that seed yields were higher in field pea than canola over two growing seasons. Likewise, we found that on a per plant basis inoculated pea seed yields were 36% greater than canola seed yields. Canola allocated 14.5% of its total biomass to seed production and 18.7% to roots, similar to the Gan et al. (2009a; 2009c) report of canola seed and roots comprising 18 and 22% of total plant biomass, respectively. In contrast, pea allocated approximately 50% of its biomass to seed production and only 4% to root production. Moreover, root biomass of canola was an order of magnitude higher than in pea. Given that this was a pot study, where root to shoot ratios tend to be lower than in the field (Wichern et al., 2008), root biomass of both crop species was likely less than what would be found under field conditions and such a marked difference between the two crop species might not be expected. Indeed, Gan et al. (2009c) found that canola root biomass was 75 to 239% greater than pea root biomass during a two-year field study, where canola was planted at a density 50% higher than pea (3 *vs*. 2 plants per 15 cm diameter lysimeter).

5.6.2. ^{15}N enrichment, recovery, and distribution in the plant-soil system

Similar amounts of ^{15}N-labeled urea were applied to pea and canola, resulting in an application rate of ^{15}N that was 2.8, 2.3, and 3.3% of total plant N uptake for canola, N-fertilized pea, and non-fertilized pea, respectively. Mayer et al. (2003b) applied ^{15}N as urea at a rate that was 2.5% of plant N uptake and was in concert with plant demand. Additions of ^{15}N-labeled urea at these rates resulted in root atom% ^{15}N excess values in pea (0.70-1.03 atom% ^{15}N excess) and canola (0.92 atom% ^{15}N excess) that were similar to those previously reported using either leaf or stem feeding of urea in field pea (Mahieu et al., 2009a; Mayer et al., 2003b; Wichern et al., 2007a; Wichern et al., 2007b), chickpea (Yasmin et al., 2010), faba bean (López-Bellido et al., 2011; Mayer et al., 2003b), white lupin (Mayer et al., 2003b), and oat (Wichern et al., 2007a; Wichern et al., 2007b). Even with relatively low ^{15}N enrichment in the roots (i.e., as low as 0.24 atom% ^{15}N excess), Mahieu et al. (2007) obtained reliable estimates of NdfR. In our study, N fertilization tended to reduce root atom% ^{15}N excess in pea—probably due to dilution with ^{14}N from the fertilizer, though this effect was not significant. Similarly, Mahieu et al. (2009a)

observed a decrease in root ^{15}N enrichment in both a nodulated pea genotype and its non-nodulating isoline with increasing rates of N fertilization. Bulk and rhizosphere soil ^{15}N-enrichment levels in this study were above natural abundance levels for both pea and canola and fell within range of those previously reported in studies using shoot or leaf ^{15}N labeling (Mahieu et al., 2009a; Mahieu et al., 2007; Yasmin et al., 2010). Thus, we were confident that the ^{15}N enrichment of the soil was sufficient to trace the root-derived N in the soil.

Recovery of ^{15}N applied using the cotton-wick method ranged between 77.4% in canola to 87.7% in N-fertilized pea. Previous pot and field studies reported ^{15}N recoveries ranging from 59 to 108% using the cotton-wick method (Mayer et al., 2003b; Wichern et al., 2007a; Wichern et al., 2007b). Incomplete recovery of ^{15}N may be partially attributed to the retention of ^{15}N in the wick-labeling system and to additive experimental errors in determining ^{15}N in the numerous plant parts used to calculate the total ^{15}N mass balance (Mayer et al., 2003b). In addition, losses of ^{15}N may be due to gaseous loss via denitrification, volatilization of ^{15}N-NH_3 from leaves (Zebarth et al., 1991), or during recovery of rhizosphere soil by drying the soil-water slurry. Recovery of ^{15}N tended to be less for canola than for pea. Canola was harvested approximately one month after pea because of differences in maturation rates, and as a result the ^{15}N in the canola-soil system was susceptible to losses for a longer duration than pea.

Heterogeneous distribution of ^{15}N within the plant system is commonly observed in studies using ^{15}N shoot- or leaf-labeling (Mayer et al., 2003b; Russell and Fillery, 1996b; Wichern et al., 2007a; Wichern et al., 2007b; Yasmin et al., 2010). As expected, aboveground plant parts were preferentially enriched in ^{15}N for both pea and canola, while ^{15}N enrichment in roots was relatively low. The distribution of ^{15}N in canola roots more closely resembled overall N distribution compared to pea roots. Presumably, the low ^{15}N distribution to roots relative to aboveground plant components is due to the direct application of the ^{15}N-urea into the stem of the plant (Wichern et al., 2010). Wichern et al. (2011) demonstrated that ^{15}N distribution was homogeneous in plant parts of pea and white lupine when ^{15}N was assimilated naturally via root uptake, whereas ^{15}N homogeneity was not achieved when plant stems were supplied with ^{15}N-urea using the cotton-wick technique. In addition, because ^{15}N labeling did not begin until the ~6 leaf stage, the duration of labeling encompassed a time when roots are a source of endogenous N for aerial parts (Malagoli et al., 2005; Schiltz et al., 2005); therefore, allocation of ^{15}N to aboveground parts is promoted further. The uneven distribution of ^{15}N between aboveground and

belowground plant parts is one of the inherent limitations of using ^{15}N shoot labeling techniques to track the fate of N from rhizodeposits, roots, and aboveground crop residues to soil following growth of the ^{15}N-labeled plant. Moreover, heterogeneous distribution of ^{15}N within plant roots can occur (Khan et al., 2002b), violating one of the assumptions of the calculation and resulting in errors in the estimation of NdfR. Estimates may be further confounded since rhizodeposition can vary spatially along the root system (Dennis et al., 2010).

Nitrogen-15 was supplied to the plants nearly continuously over the course of five weeks in an attempt to maintain a relatively constant ^{15}N enrichment of roots—as opposed to supplying the ^{15}N in infrequent pulses. Indeed, continuous labeling has been proposed as a means of minimizing the heterogeneity of ^{15}N enrichment among all plant parts and between roots and rhizodeposits (Mahieu et al., 2009a). Mahieu et al. (2009a) observed a slightly better relationship between belowground N and plant N when pea plants were labeled continuously (r^2=0.80) rather than fortnightly (r^2=0.73). Moreover, ^{15}N labeling did not encompass the full duration of root growth; i.e., ^{15}N labeling began at the 5–7 leaf stage and ended well into the flowering stage for both plants. Flowering tends to mark a decrease in root growth in pea and the onset of root loss (Gan et al., 2010b; Gan et al., 2009c; Gavito et al., 2001; Voisin et al., 2002a); therefore, the ^{15}N diluting effect of further root growth was probably not severe for pea. In contrast, root biomass and N yield in canola peaks at late pod filling (Gan et al., 2010b; Gan et al., 2009c), suggesting that N rhizodeposition may be overestimated for canola. Nevertheless, the relative distribution of ^{15}N and total plant N followed a similar pattern among the crop treatments; therefore, the relative differences in the estimates of N rhizodeposition among the crop treatments are probably realistic.

5.6.3. Effect of N fertilization on biomass and N partitioning in pea

Nitrogen fertilizer enhanced aboveground biomass and N content of pea by 38% and 43%, respectively. The high response of pea to N fertilizer relative to inoculated plants suggests that N fixation was not optimal in this system. Mahieu et al. (2009a) found that total dry weight, root to shoot ratios, and plant N content increased with level of N fertilization in a nodulating pea and its non-nodulating isoline. In the nodulating pea, high levels of N suppressed N_2 fixation and increased production of foliar and seed biomass, but did not increase root biomass relative to the same nodulating pea genotype that did not receive supplementary N (Mahieu et al., 2009a).

Similarly, in our study, the application of urea fertilizer (120 kg N ha^{-1}) increased aboveground biomass production of non-inoculated pea relative to the inoculated pea, particularly in seed, with no significant increase in root biomass. This is in contrast to Voisin et al. (2002a) who found that increased mineral N availability increased shoot and root biomass of pea, but did not have an effect on seed biomass or N yields. Even with moderate N fertilization of nodulating pea, total biomass and N content increased relative to non-fertilized pea (Mahieu et al., 2009a).

Distribution and recovery of ^{15}N was not influenced by N fertilization in pea. In both N fertility treatments, 93% of recovered ^{15}N was partitioned in aboveground plant components, while 7% was found in roots and soil. These results closely coincide with those found by Mahieu et al. (2009a), who reported that—regardless of N fertilizer application rate to the nodulating pea genotype—92% of recovered ^{15}N was distributed in aboveground plant components and 8% was distributed in roots and soil. The effect of N fertilizer on N allocation and ^{15}N distribution of pea cannot be clearly differentiated since the N-fertilized pea was not inoculated. Nevertheless, our results are similar to those of Mahieu et al. (2009a) who reported no difference in the distribution of recovered ^{15}N in nodulated pea and non-nodulated pea at medium to high N-fertilization. While we observed no significant difference in the partitioning of N rhizodeposition to total plant N with the addition of N fertilizer (14.4 *vs*. 17.5%), Mahieu et al. (2009a) found that N rhizodeposition as a percentage of total plant N tended to increase from 2.7 to 5.5% between non-N-fertilized and high N-fertilized pea plants.

5.6.4. Significance of belowground N to total remaining residue N—quantity of N rhizodeposition

Considering the removal of N from the soil-plant system following seed harvest, belowground contributions from N rhizodeposition and roots comprise a significant return of N to the soil. Indeed, including NdfR values in the estimate of the overall plant-N balance for canola indicates that NdfR, roots, and aboveground straw (leaves, stems, and pods) comprised 52%, 16% and 32% of the crop residue remaining after seed harvest. In pea, NdfR comprised 49% of the crop residue remaining after seed harvest, while root and aboveground residues (leaves, stems, and pods) represented 12% and 39%, respectively. Belowground N comprised 22% of total plant N for inoculated pea, which was within range (14-41%) of that reported in previous pot and field studies (Jensen, 1996c; Mayer et al., 2003b; Wichern et al., 2007a).

Differences in the contribution of NdfR to the total plant N balance in the literature may be attributed to differences in the choice and implementation of labeling techniques, including differences in ^{15}N labeling duration, frequency, and ^{15}N urea concentration (Mahieu et al., 2009a). Moreover, experimental conditions, including growth conditions, nutrient and water availability, soil type, and plant genotype contribute to the wide range of results reported (Wichern et al., 2008). In our study, the relatively small pot size used may have promoted foliar growth over root growth, thus resulting in a lower NdfR compared to what might occur in the field, where root growth is not restricted and biomass is expected to be higher (Mayer et al., 2003b).

Canola has a high requirement for N and therefore relies on N fertilizer to maintain adequate seed yields and quality (Grant and Bailey, 1993). Assessing NdfR can assist in resolving whether changes in soil N under canola are a result of residual fertilizer N (i.e., poor fertilizer use efficiency) or the recycling of N through the canola root biomass. Canola contributed 29.4% of its total N to soil through N rhizodeposition by the time the plant reached maturity, with the N in intact canola roots comprising a larger proportion of total plant N than pea. In a non-^{15}N labeling field study, Gan et al. (2010b) found that by maturity, the average amount of root N in canola was 21 kg N ha^{-1} and 16 kg N ha^{-1} for pea. Prior to harvest, canola root N peaked at the late pod stage at approximately 31 kg N ha^{-1}, while pea root N peaked earlier at 25 kg N ha^{-1} at the late flowering stage. The greater input of N by canola in the field study by Gan et al. (2010b), particularly at growth stages preceding physiological maturity, supports the high amount of root-derived N (mg N pot^{-1}) recovered in soil at maturity in this study.

Canola produces a high proportion (85%) of its total root length as 'extra fine' (<0.4 mm dia.) roots, while this proportion is only about 50% for pea (Liu et al., 2010). As a result, fine roots of canola probably contributed to the calculated N rhizodeposition values to a greater extent than for pea, despite attempts at careful hand-collection of roots from the bulk soil. Moreover, in bulk soil, where fine roots were more likely to be missed during sampling, atom% ^{15}N was highest for canola. In contrast, atom% ^{15}N values in the rhizosphere soil fraction were highest for pea. Because root atom% ^{15}N values were similar between pea and canola, the differences in atom% ^{15}N observed in bulk *vs*. rhizosphere soils may be due to a higher contribution of root exudates and sloughed root cells to N rhizodeposition in the case of pea and

a higher contribution of fine roots in the case of canola; whether this was the case, however, could not be definitively determined. Further research involving shorter-duration ^{15}N labeling experiments may help to distinguish between the modes of root-derived input of N to soil between these two crop species.

5.6.5. Contribution of N rhizodeposition to soil inorganic N pools—quality of N rhizodeposition

Inorganic N comprised 4.3%, 12.9%, and 13.8% of total N rhizodeposition in soil cropped with canola, N-fertilized pea, and non-fertilized pea, respectively. These values are lower than those reported by Mayer et al. (2003b) and Wichern et al. (2007a), where inorganic N comprised 25% and 32% of total NdfR in pea, respectively. This indicates that a higher proportion of NdfR in our study was found in soil organic N fractions and microbial biomass and had not mineralized to the same extent. Jensen (1996c) found that amounts of inorganic N were too low to measure the contribution of rhizodeposition to inorganic N pools and that the majority of N rhizodeposition was in the organic N fraction following harvest of barley and pea. Inorganic N was detected at harvest of both pea and canola; however, the relatively lower NO_3^--N and NH_4^+-N in soils grown with canola in our study was likely due to a higher demand for N compared to pea. Similarly, soil NO_3^--N was greater under pea than canola over the growing season, except at the seedling stage when N demand was lower (Gan et al., 2010b). The lack of difference in inorganic N between the non-fertilized and fertilized pea indicates that the potential for an N sparing effect due to enhanced reliance on N fixation in the non-fertilized pea was likely low. Despite the supply of N fertilizer in canola, little root-derived N contributed to the inorganic N pool and there was no significant difference between non-fertilized and N-fertilized pea. Conversely, Janzen (1990) reported that a higher rate of N fertilization applied to wheat increased the proportion of root-derived N in the inorganic form to 46%, possibly due to excess N accumulation in the plant, which was not likely the case for either canola or pea since the proportion of root-derived N in inorganic N forms was relatively low (4-13%).

The higher contribution of inorganic NdfR to total N rhizodeposition in soils grown with pea may be due to higher release of inorganic N directly or to differences in mineralization of root-derived organic N. Direct release of NH_4^+ may be significant, but can differ among crop species (Brophy and Heichel, 1989). While we did not directly determine the composition of

rhizodeposits, the data suggest that the compounds that comprise root-derived N in pea are more labile, and hence more susceptible to mineralization, than those derived from canola roots. In the field, Gan et al. (2010b) found that net N mineralization was 1.5 times greater under pea than canola. Conversely, there was no difference in net N mineralization from the soils containing residual pea and canola roots (Engström and Lindén, 2012). Soon and Arshad (2002) found that 38% of the initial N in both pea and canola roots was mineralized over the course of 10 to 11 months during the first year of a field study; however, when the experiment was repeated the following year, 64% of the initial N from pea roots was mineralized compared to only 22% from canola roots. While the results of our study suggest that N mineralization from rhizodeposits is higher in pea than canola—at least within the initial growing season—further research is required to determine whether this difference is sustained following harvest.

5.7. Conclusion

Rhizodeposited N comprised a significant proportion of total plant N, and included root-derived N released to soil from living and decaying roots prior to harvest for both pea and canola. Moreover, N rhizodeposition comprised the majority of belowground crop residue N for both pea and canola. While on a per plant basis total N rhizodeposition was greater for canola than for pea, the greater proportion of root-derived N in soil inorganic N pools to the total root-derived N in pea suggests a difference in quality between the root-derived residues of these two crop species. Further research is required to resolve the source of root-derived N to soil between these two crop species; specifically, to determine whether fine roots of canola contributed more to N rhizodeposition in comparison to pea. Our study, as well as previous research highlights the importance of N rhizodeposition to the total plant N balance of pea. This study is the first to quantify N rhizodeposition of canola and will help to further our understanding of the influence of canola residues on soil N dynamics.

6. DIFFERENTIATING BETWEEN THE SUPPLY OF N TO WHEAT FROM THE ABOVEGROUND AND BELOWGROUND RESIDUES OF PRECEDING CROPS OF PEA AND CANOLA

6.1. Preface

Most studies that examine the fate of crop residue N in the soil system and the supply of residue-derived N to succeeding crops do not account for the contribution of N rhizodeposition. In the previous study, I demonstrated that root-derived N comprised ~50% of the total residue-N remaining following seed harvest of pea and canola. Whereas root-derived N from canola comprised a greater input of N to soil compared to pea, a higher proportion of root-derived N from pea contributed to the soil inorganic N pool. Therefore, the contribution of total residue derived N, including rhizodeposits, to a succeeding crop may differ between these two crop species due to differences in the quantity of N remaining and quality of the residues. Previous research has shown that N mineralization between straw and root residues may vary within a single crop species, therefore aboveground and belowground residues may contribute N to a succeeding crop to varying degrees. The application of ^{15}N labeled crop residues provides a direct method to determine the fate of residue-derived N in soil and to a succeeding crop. Using a cross ^{15}N labeling approach, the work described in this chapter was carried out to differentiate between the supply of N from the aboveground and belowground residues of pea and canola to a succeeding wheat crop.

6.2. Abstract

The supply of nitrogen (N) from crop residues to succeeding crops can differ due to differences in the quantity of N in the residues as well as the susceptibility of those residues to mineralization and synchronization with plant demand. Furthermore, root residues may have different mineralization characteristics from corresponding straw residues within a single crop species. Nitrogen rhizodeposition comprises an important component of the total residue N balance, but the N from this pool is often not included in studies that use ^{15}N approaches to trace residue-derived N in succeeding crops. The objective of this study, therefore, was to differentiate

between N supply from aboveground and belowground crop residues, including rhizodeposits, of pea and canola to wheat using ^{15}N labeling. Specifically, a cross ^{15}N-labeling approach was used such that wheat was grown on ^{15}N-labeled belowground residues and non-labeled aboveground residues and vice versa. On average, N derived from belowground residues in wheat was almost twice that from aboveground residues. In total, the contribution of N to wheat from residues was 5.4%. Higher input of both aboveground and belowground residue N tended to increase the residue-derived N in wheat from canola (6.4%) relative to pea (5.0%). However, differences in the percent recovery of ^{15}N based on the amount of residue-^{15}N initially applied revealed that a higher proportion of belowground residue N from pea (13.1%) was recovered in wheat compared to the corresponding aboveground residue N from pea (9.6%), and both aboveground and belowground residue N from canola (7.3 and 6.5%, respectively). The total supply of N to wheat from preceding pea and canola crops was relatively low, likely due wide C to N ratios. This study demonstrates the importance of belowground N to the supply of N to succeeding crops.

6.3. Introduction

The crop residues that remain following seed harvest comprise the major input of organic materials and organic sources of N to soil in cropping systems that receive limited inputs of manure. The biochemical composition of crop residues are important factors in determining the fate of residue-derived N in mineralization-immobilization processes (Abiven et al., 2005; Trinsoutrot et al., 2000) and in processes further along the N cycle such as denitrification (Huang et al., 2004). Residue quality parameters, such as N concentration, lignin and polysaccharide content, and C to N ratio, can vary markedly among crop species, resulting in varied fates of N from the residues of different crops. For example, Soon and Arshad (2002) found that, averaged over two years, net N mineralization from straw residues was 5.6, 0.7, and 0 kg N ha^{-1} for pea, canola, and wheat, respectively. Conversely, Lupwayi et al. (2006) found that a greater amount of N was released from the decomposition of canola residue (10 to 25 kg ha^{-1}) compared to pea (4 to 18 kg ha^{-1}) and wheat residues (2 kg ha^{-1}). Furthermore, they found that a higher proportion of the N in canola residues was released in comparison to pea.

The influence of aboveground crop residues on soil N dynamics has been studied extensively (Janzen and Kucey, 1988; Jensen, 1994a; Lupwayi and Soon, 2009; Lupwayi et al., 2006). Quantification of root N and its contribution to soil N processes also have been examined

(Abiven et al., 2005; Jensen, 1994b; Jensen, 1996b; Soon and Arshad, 2002; Trinsoutrot et al., 2000). These studies revealed that the decomposition and N mineralization patterns of aboveground crop residues do not necessarily coincide with those of the corresponding roots (Soon and Arshad, 2002; Trinsoutrot et al., 2000). Soon and Arshad (2002) found that N mineralization from canola and pea roots was similar, despite pea roots having a much narrower C to N ratio than canola roots. Conversely, N mineralization from pea straw was greater than from canola straw and reflected their different C to N ratios (Soon and Arshad, 2002), indicating that the factors controlling N mineralization between aboveground and root residues vary within and among crop species. That is, quality parameters other than C to N ratio may have determined the similar N mineralization from canola and pea roots. In addition, quality parameters (e.g., C:N, N concentration, polyphenol, cellulose, and lignin content) of aboveground plant components are not the same as those of roots, even within the same plant (Abiven et al., 2005; Trinsoutrot et al., 2000). Therefore, roots comprise an important component of the residue N that may influence the supply of N to succeeding crops. Nitrogen-15 techniques can be used to study the supply of residue-N to a succeeding crop relative to the supply from indigenous soil inorganic and fertilizer N.

Direct labeling of crop residues with ^{15}N provides a better estimate of the contribution of residue-N to subsequent crops than indirect ^{15}N isotope dilution and A-value approaches (Hood et al., 1999), which tend to overestimate residue-derived N (Hood et al., 1999) and give variable and sometimes negative results (Stevenson et al., 1998). On the other hand, estimates from A-value and isotope dilution methods incorporate the contribution of belowground N to succeeding crops (Stevenson et al., 1998), which is an advantage of these methods. Direct labeling is not without its limits either, as an underestimation of N contribution can occur if there is pool substitution. That is, if inorganic ^{15}N released from mineralized residues is immobilized and substituted with ^{14}N that is concurrently released by mineralization of soil organic matter, recovery of ^{15}N from the labeled residue is reduced (Stevenson et al., 1998). Following a comparison of both direct and indirect ^{15}N-labeling methods (Hood, 2001; Hood et al., 2000; Hood et al., 1999), Hood (2001) suggested that direct labeling should be the standard method against which indirect methods ought be evaluated. In most direct labeling studies, plants assimilate ^{15}N via root uptake from labeled fertilizer that is supplied to the growth medium. The aboveground residues, and sometimes the roots, are harvested and incorporated into the soil to

which the subsequent crop is planted; i.e., separate from the soil in which the ^{15}N-labeled plants were grown. The limitation to this approach is that by removing the plant from the soil in which it is grown, N rhizodeposits are not included and therefore an unknown proportion of residue-N does not interact with the following crop. To address this issue, ^{15}N shoot, petiole, or leaf labeling is sometimes used to label the whole plant with ^{15}N, including rhizodeposits. A second crop is grown in the same pot and ^{15}N mineralized from above and belowground residues—including the rhizodeposits—are traced in the second crop.

Despite the importance of rhizodeposition to the total residue-N balance, to date only a few studies have examined the fate of residue-N (including N rhizodeposition) to succeeding crops using direct ^{15}N labeling (Lam et al., 2012; Mayer et al., 2003a; McNeill and Fillery, 2008; McNeill et al., 1998; Russell and Fillery, 1996a). Mayer et al. (2003a) found that crop residues from ^{15}N-labeled pea, faba bean, and white lupin contributed 18, 42, and 32% of total plant N accumulated in a subsequent wheat crop. Whereas their estimates included N rhizodeposition, the authors did not distinguish between above and belowground residues. McNeill et al. (1998) found that 25 and 18% of ^{15}N-labeled subterranean clover and serradella belowground residues were recovered in a succeeding wheat crop, but did not determine the contribution of N in aboveground residues. Similarly, Russell and Fillery (1996a) examined the fate of belowground residue N from ^{15}N labeled plants without consideration for the input of N from aboveground residues. However, both aboveground and belowground residues should be present to trace the fate of residue N to subsequent crops since this would be the case in practice. To date, no studies have differentiated between the supply of N from belowground and aboveground crop residues to a succeeding crop using a direct ^{15}N labeling approach.

Shoot ^{15}N-labeling generally results in a non-homogenous distribution of ^{15}N in the above and belowground plant components (Wichern et al., 2011). However, uniform labeling of crop residues with ^{15}N is needed in order to track the whole-residue N contribution to both the soil and the succeeding crops (Fillery and Recous, 2001). Compounds that comprise the crop residue material may decompose at different rates and to varied extents (Trinsoutrot et al., 2000); therefore, the contribution of ^{15}N to the inorganic N pool can differ, which would result in erroneous estimates of residue-N contribution to the plant. Moreover, plant residues labeled using shoot ^{15}N methods may underestimate the contribution of belowground residue N to succeeding crops because the aboveground residues are often more enriched in ^{15}N and, as

outlined in the examples above, do not necessarily share similar decomposition characteristics as roots. However, Mayer et al. (2004) reported that crop residues, including roots, had similar decomposability as rhizodeposits. A cross-^{15}N application approach—in which ^{15}N-labeled aboveground residues is applied to non-labeled belowground residues and vice versa—was suggested to differentiate the N supply of aboveground residues from root residues (Mayer et al., 2003a). In this way, the excess ^{15}N is in either the aboveground or belowground residues, but not both. Taking this approach, the objective of the present study was to differentiate between the supply of N from aboveground (AG) and belowground (BG) residues of canola and pea to a subsequent wheat crop using cross-^{15}N labeling.

6.4. Materials and Methods

6.4.1. ^{15}N-residue preparation and application

The first phase of the experiment involved generating ^{15}N-labeled and non-labeled pea and canola residues. Two sets of pea (*Pisum sativum* cv. CDC Meadow) and canola (*Brassica napus* cv. 45H21) plants were grown, simultaneously, in pots (20-cm i.d., 20-cm deep) containing a soil-sand mixture (1:1 w/w) in a greenhouse. Soil characteristics and growth conditions are described in detail in Chapter 5 (section 5.4). In both sets of pea and canola, plants in half the pots were labeled with ^{15}N-urea using the cotton-wick technique described in Chapter 5 (section 5.4). The remaining canola and pea plants were grown without ^{15}N-labeling. At maturity, one set of pea and canola plants was destructively sampled; after harvesting the seed, the aboveground residues from the second set of ^{15}N-labeled pea and canola plants were swapped with those from the non-labeled plants (Fig. 6.1). The residues were incorporated into the soil and the pots were then seeded with wheat (*Triticum aestivum* cv. AC Barrie).

Chapter 5 (section 5.5) reports on the plant N uptake, N rhizodeposition, and ^{15}N enrichment data collected from the first set of pea and canola plants, which were used to estimate the amount of N (mg pot^{-1}), the C to N ratio, and the atom% ^{15}N excess of the AG and BG crop residues applied in the wheat-phase of the experiment (Table 6.1). The AG biomass of the second set of ^{15}N-labeled and non-^{15}N-labeled pea and canola plants was harvested at maturity and the seed removed; the AG crop residues—including leaves, stems, and pod walls—were dried at 60°C, weighed, and then coarsely chopped (1–3 cm). The soils and roots from this

second set of plants remained intact in the pots and were stored at 2°C until processing and incorporation of the AG crop residue.

Total AG biomass within each crop treatment was ranked by weight for both the ^{15}N-labeled and non-labeled plants. These rankings determined the cross-application of AG residues to the BG residues; for example, the pot containing the ^{15}N-labeled BG residues from the ^{15}N-labeled plant having the heaviest AG biomass received the non-^{15}N-labeled AG residues from the non-^{15}N-labelled plant having the heaviest AG biomass and vice versa. This was repeated for the ^{15}N-labeled and non-^{15}N-labeled plants having the next heaviest AG biomass, and so on. The amount of ^{15}N excess from BG residues (roots and rhizodeposits) from ^{15}N-labeled plants was estimated from root biomass and atom% ^{15}N excess data in roots and soils collected from the set of ^{15}N-labeled pea and canola plants that were destructively sampled. The AG additions of ^{15}N were estimated more precisely because the exact weight of the biomass for each AG component harvested was determined directly.

The AG residues were incorporated into the soil in the pots containing the BG residues (roots and rhizodeposits) in accordance with the cross-application scheme described in the preceding paragraph. Each pot contained both AG and BG residues, but with the ^{15}N label located either in the AG or the BG residues—never in both (Fig. 6.1). Following incorporation, the pots were placed in a chest freezer at -15°C for two weeks to simulate a winter period. The pots were removed from the chest freezer and placed on a bench in the greenhouse for one-and-a-half weeks prior to planting the wheat crop.

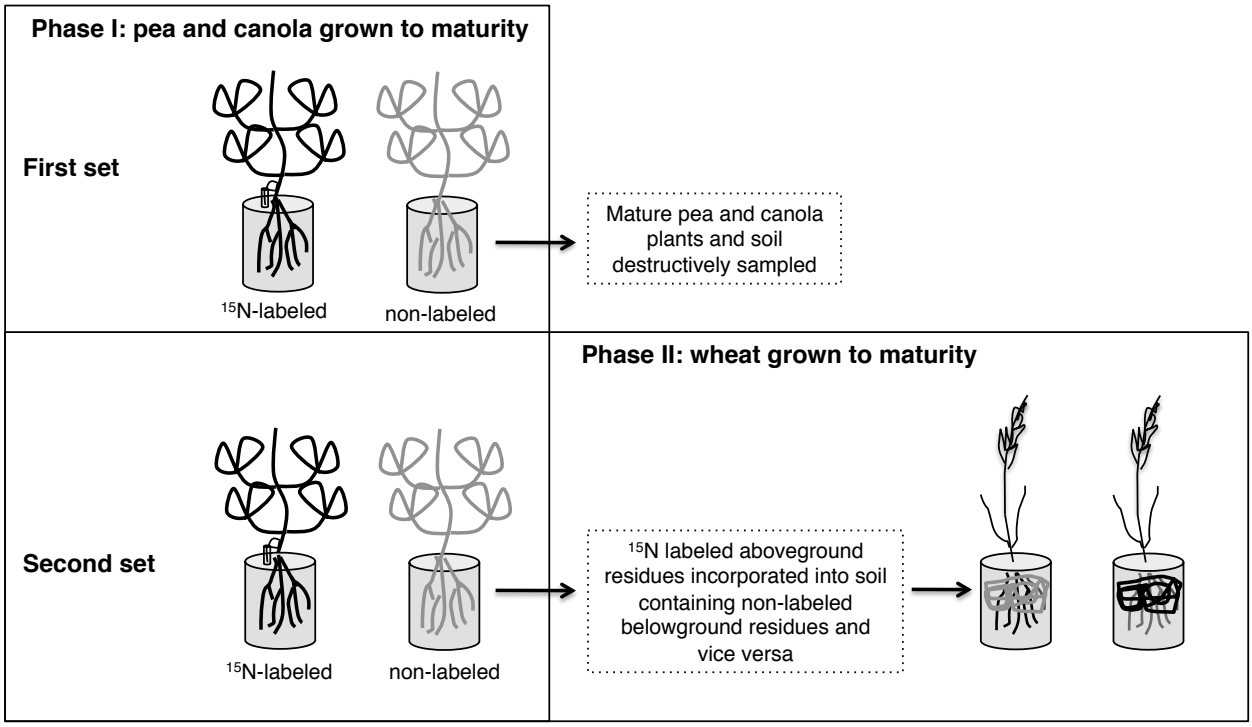

Fig. 6.1. Schematic of experimental setup. Two sets of ^{15}N-labeled (black) and non-labeled (grey) pea and canola plants were grown to maturity during Phase I. At maturity, plants in the first set were destructively sampled and analyzed. In the second set of plants, the ^{15}N-labeled aboveground residues (black) were incorporated into the soils containing non-labeled (grey) roots and rhizodeposits of the mature pea and canola; likewise, the non-labeled aboveground residues (grey) were incorporated into the soils containing ^{15}N-labeled (black) roots and rhizodeposits. Phase II of the experiment consisted of growing wheat in the pots containing the ^{15}N cross-labeled aboveground and belowground residues of pea and canola.

Table 6.1. Mean ± standard deviation (n=8) of application rates of N (mg pot^{-1}), C to N ratios, and ^{15}N enrichment (atom% ^{15}N excess) of ^{15}N-labeled belowground† (BG) residues and ^{15}N-labeled aboveground (AG) residues of mature canola, N-fertilized pea, and non-fertilized pea plants grown in a greenhouse that were mixed into the soil preceding wheat growth.

Residue	Canola	Pea N-fertilized	Pea non-fertilized
		N application rate (mg N pot^{-1})	
Roots	17.9 ± 1.4	6.8 ± 2.4	5.5 ± 0.9
NdfR	59.6 ± 21.7	28.8 ± 7.8	23.7 ± 5.8
BG total	77.5 ± 21.8	35.6 ± 8.0	29.3 ± 6.4
AG straw	31.8 ± 7.3	22.8 ± 4.5	19.2 ± 4.4
		C:N‡	
Roots	79 ± 9	24 ± 2	23 ± 3
AG straw	148 ± 24	85 ± 10	83 ± 16
		Atom% ^{15}N excess	
Roots and rhizodeposits§	0.924 ± 0.086	0.699 ± 0.185	1.027 ± 0.341
AG straw	1.881 ± 0.238	2.128 ± 0.589	2.915 ± 0.915

† Belowground residues refers to roots and N derived from rhizodeposition (NdfR) determined at maturity of canola, N-fertilized pea, and non-fertilized pea

‡ C to N ratios of roots and straw calculated based on C concentration of 42% (Gan et al., 2009b)

§ ^{15}N enrichment of roots and rhizodeposits assumed to be equal

6.4.2. Wheat growth and harvest

Five seeds of red spring wheat were sown into each pot containing either the ^{15}N-labeled AG residues plus the non-labeled BG residues or the non-labeled AG residues plus the ^{15}N-labeled BG residues of canola, N-fertilized pea, and non-fertilized pea on March 18, 2011. The wheat was thinned to two plants per pot upon emergence. All plants were supplied with 20 kg P ha^{-1} as KH_2PO_4 (32 mg P $plant^{-1}$) and 120 kg N ha^{-1} as urea (192 mg N $plant^{-1}$) at planting. To ameliorate visible signs of nutrient deficiency, each pot received 5-g of Nutricote (13-13-13) with micronutrients on April 25, 2011. Plants were watered frequently with deionized water. The pots were arranged as a randomized complete block design with eight replicate pots for each treatment for a total of 48 pots (3 crop residues x 2 ^{15}N-residue types x 8 replicates). All wheat plants were harvested at maturity (94 DAS; June 20, 2011) and the aboveground plant materials separated into grain and straw. Crown roots and large root fragments were recovered from the soil. Immediately after harvest, subsamples of the soil (80 g) were collected and stored in 50-mL Falcon tubes and frozen at -20°C until they were analyzed for inorganic N and ^{15}N. Plant materials were dried at 60°C; the remaining soils used for total N and ^{15}N analysis were air-dried. The dried plant and soil samples were finely ground in a ball mill.

Soil NO_3^--N and NH_4^+-N were determined by extraction with 2.0 *M* KCl followed by colorimetric determination of NO_3^--N and NH_4^+-N using a Technicon AutoAnalyzer (Labtronics Inc., Tarrytown, NY). The ^{15}N-enrichment of the NO_3^- and NH_4^+ pools was determined by analyzing the 2.0 *M* KCl extracts using the acidified diffusion disk technique described by Stark and Hart (1996).

Diffusion disks and the finely ground plant and soil samples were weighed into tin capsules and analyzed for N concentration (%) and $\delta^{15}N$ using an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific Inc., Waltham, MA) coupled to an elemental analyzer (Costech ECS4010. Costech Analytical Technologies, Inc., Valencia, CA). The average atom% ^{15}N value (0.36898 atom% ^{15}N) from non-labeled control soils was used to calculate atom% ^{15}N excess values (Mayer et al., 2003b).

6.4.3. Calculations

The percentage of N-derived from residues (%Ndfr) in the wheat-soil system was calculated according to Hauck and Bremner (1976):

$$\%\text{Ndfr} = \frac{\text{atom\% } ^{15}\text{N excess wheat}}{\text{atom\% } ^{15}\text{N excess residues}} \text{ x } 100 \quad [6.1]$$

Percent Ndfr was calculated for both AG and BG residues; for calculations of %Ndfr from BG residues, it was assumed that the atom% ^{15}N of the rhizodeposits was equal to that of the pea and canola roots. The amount of Ndfr in the wheat-soil system was calculated by multiplying %Ndfr by the total N (mg pot^{-1}) in wheat.

6.4.4. Statistics

Statistical analyses were performed using SPSS® Statistics version 20.0 for Mac (IBM Corp., 2011). Two-way analysis of variance was performed with ^{15}N-residue (AG or BG) and crop treatment (canola, pea +N, and pea -N) as fixed effects and block as a random effect. Normality of residuals was tested using the Shapiro-Wilk statistic and homogeneity of variances was tested using Levene's test. Where necessary, data were log transformed to meet the assumptions of the ANOVA. Means comparisons of main effects were made using Tukey's Honestly Significant Difference test. Orthogonal contrasts were used to compare specific means when a significant interaction between main effects occurred. All tests were declared significant at $P \leq 0.05$.

6.5. Results

6.5.1. Wheat biomass and N accumulation

Wheat produced 14% more grain when grown on N-fertilized pea residue compared to canola residue (P=0.032; Fig. 6.2). However, there was no difference in grain biomass when wheat was grown on the residue of non-fertilized pea compared to N-fertilized pea or canola. Wheat grown on pea residues (N-fertilized or unfertilized) yielded about 24% more total biomass (grain, shoots, and roots; P<0.001) than wheat grown on canola residues. Despite the lower grain biomass, the N concentration (%) in wheat grain was significantly greater when grown on the residues of canola compared to pea (P=0.010; data not shown). As a result, there was no effect of previous crop residues on grain N (P=0.333, Fig. 6.2). There also was no significant effect of previous crop species on total wheat N accumulation (P=0.397). The location of the ^{15}N label (i.e., either in AG or BG) had no effect on wheat biomass or N accumulation (P>0.05).

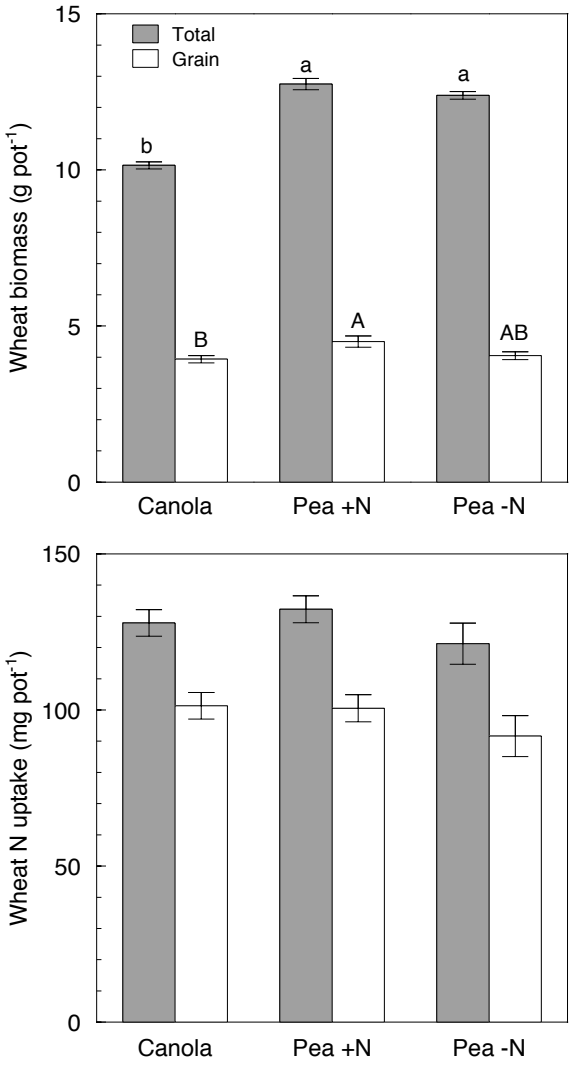

Fig. 6.2. Grain and total plant biomass (g pot^{-1}) and grain N and total plant N (mg N pot^{-1}) of mature wheat grown in a greenhouse and amended with either ^{15}N-labeled aboveground or belowground residues of canola, N-fertilized pea, and non-fertilized pea. Bars indicate standard error of the mean (n=16) and same letters above means indicate no significant difference among treatments according to Tukey's HSD (*P*>0.05).

6.5.2. Recovery of ^{15}N and contribution of residues to a subsequent wheat crop

Recovery of ^{15}N in wheat was significantly influenced by the location of ^{15}N in the residue type (AG *vs*. BG; P=0.022) as well as crop treatment (P<0.001). A higher percentage of ^{15}N from pea BG residues (13.1%) was recovered in wheat compared to canola BG residues (6.5%; Fig. 6.3). Similarly, the recovery of ^{15}N in wheat from the AG residues of pea tended to be greater than that of canola (9.6 *vs*. 7.3%), but the difference was not significant (P=0.078). Total recovery of ^{15}N in the wheat-soil system was significantly greater when the ^{15}N label was in the BG residues of pea as compared to the BG residues of canola (Fig. 6.3). The high recovery (>100%) of ^{15}N from the BG residues of pea indicates that the initial amount of ^{15}N in the residues was likely underestimated. On the other hand, the low recovery (73%) of ^{15}N from the BG residues of canola indicates that the initial amount of ^{15}N in these residues was likely overestimated—though it also is possible that some ^{15}N was lost from the wheat-soil system. Recoveries of ^{15}N from the AG residues in the wheat-soil system also were relatively low (<60%), and there were no differences between treatments (Fig. 6.3). Most of the recovered ^{15}N was retained in the soil. The amount of ^{15}N recovered in the biomass of the wheat seedlings that were thinned from the pots was less than 1% of the ^{15}N added from the pea and canola residues (data not shown).

The percentage of wheat-N derived from residues was influenced by residue type (AG/BG; P<0.001) and crop treatment (P=0.021), though there was no significant interaction between the two factors (P=0.867). Belowground residues contributed 4.5 mg N pot^{-1}, on average, to a subsequent wheat crop, accounting for 3.6% of total wheat N—twice the amount contributed from AG residues. In total, the AG and BG residues contributed 5.4% of total wheat N averaged across residues from all previous crop treatments. When the data were analyzed within each residue type, the percentage of wheat-N derived from the AG residues of canola was greater than from pea AG residues (Fig. 6.4), though there was no significant difference in %Ndfr from the BG residues of the different crop species (P=0.496). Taken together, the %Ndfr from the AG plus BG residues of pea and canola was 5.0 and 6.4%, respectively. The total amount of residue-N in the soil following incorporation of canola residues (33.1 mg pot^{-1}) was 68% greater than when pea was the previous crop (averaged over both N treatments, data not shown). There was a significant linear relationship between the amount of AG residue N applied and the %Ndfr in wheat (P<0.001, r^2=0.47); however, no relationship could be determined between BG residue N

application and %Ndfr because the amount of belowground N was estimated rather than determined directly.

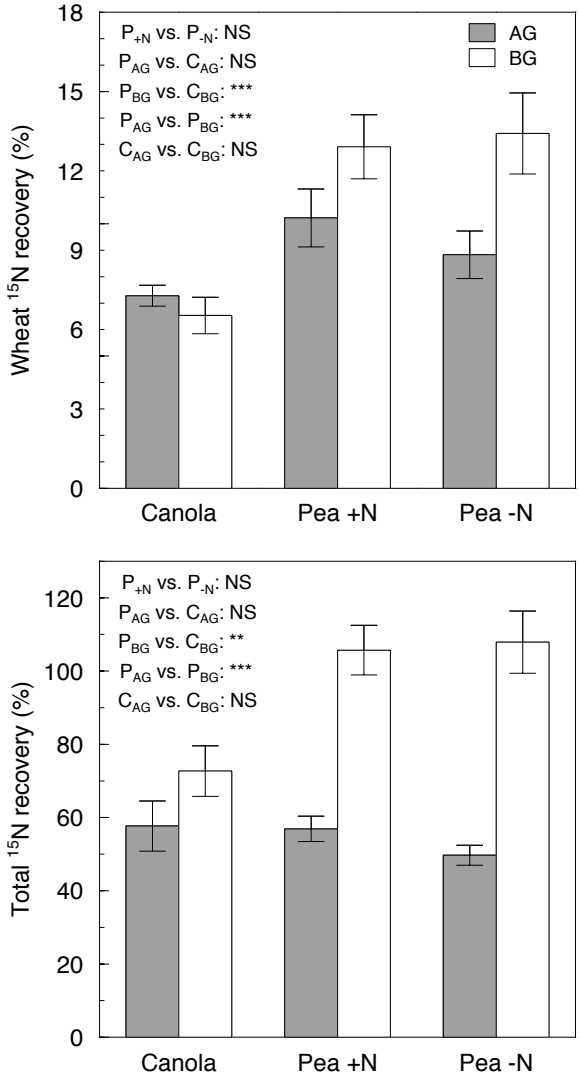

Fig. 6.3. Recovery of added ^{15}N (%) in mature wheat and total ^{15}N recovery in soil plus wheat grown in a greenhouse and amended with either ^{15}N-labeled aboveground (AG) or belowground (BG) residues of canola (C) and N-fertilized (+N) and non-fertilized (-N) pea (P). Bars represent means ± standard errors (n=8). NS, no significant difference (P>0.05), *, ** significant difference (P<0.001, P<0.01, respectively) between means based on orthogonal contrasts.**

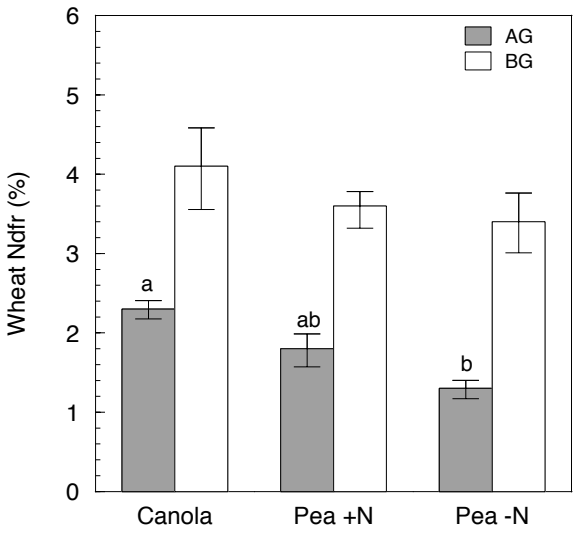

Fig. 6.4. Percentage of N derived from residues (Ndfr) in mature wheat grown in a greenhouse and amended with either ^{15}N-labeled aboveground (AG) or belowground (BG) residues of canola, pea N-fertilized (+N), and pea non-fertilized (-N). Bars represent means ± standard errors (n=8). Similar letters indicate no significant difference among the residues of crop species (*P*>0.05) according to Tukey's HSD test.

6.5.3. Residue contribution to inorganic soil N

At wheat harvest, soil NO_3^--N and NH_4^+-N were 5.8 and 12.0 mg kg^{-1}, respectively (data not shown). There was high variability for both the soil NO_3^--N (CV=52%) and NH_4^+-N (CV=79%) data and therefore no significant differences among crop treatments could be detected (*P*=0.378). The high variability in the data and the high NH_4^+-N may be partially attributable to the presence of slow release Nutricote fertilizer in the soil, which contains equal proportions of NO_3^--N and NH_4^+-N. However, %Ndfr in the NO_3^--N pool was significantly higher compared to the NH_4^+-N pool (paired t-test, *P*<0.001), indicating that the N mineralized from the crop residues was subsequently nitrified. There was a significant effect of residue type (i.e., AG or BG) on %Ndfr for NO_3^--N (*P*<0.001) and NH_4^+-N (*P*=0.031). There was also a significant effect of previous crop treatment on %Ndfr for NO_3^--N (*P*=0.003) and NH_4^+-N (*P*=0.010), but no interaction between residue type and previous crop. There was no difference in %Ndfr from BG residues among crop treatments for both NO_3^--N and NH_4^+-N pools (Fig. 6.5). The %Ndfr from AG residues was lowest under non-fertilized pea in the NO_3^--N pool and highest under canola in the NH_4^+-N pools. Less than 2.5% of the residue-^{15}N was recovered in both soil inorganic N pools (data not shown).

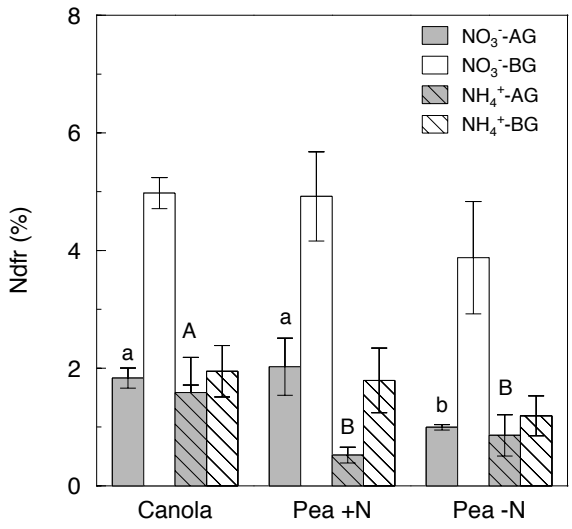

Fig. 6.5. Percentage of N derived from residues (Ndfr) in the soil NO_3^--N and NH_4^+-N pools under mature wheat as affected by the aboveground (AG) and belowground (BG) crop residues of canola (C) and N-fertilized (+N) and non-fertilized (-N) pea. Bars represent means ± standard errors (n=8). Same letters within an inorganic N pool indicate no significant difference according to Tukey's HSD test (*P*>0.05).

6.6. Discussion

6.6.1. Biomass and N uptake of a succeeding wheat crop

Pulse crops often have a positive effect on the yield of subsequent cereal crops when included in crop rotations compared to cereal monoculture (Bremer et al., 2011; Gan et al., 2003), as well as compared to oilseed crops (Gan et al., 2003). Indeed, wheat biomass yields were greater following pea relative to canola, despite the fact that canola contributed more residue-N, both from AG and BG sources. The lack of difference in wheat N uptake from the residues of the different crops indicates that the positive effect that pea had on wheat biomass was controlled by factors other than N supplied from the decomposing pea residues. In a landscape-scale field study, the non-N benefit of growing pea contributed 91% of the total yield increase of a following wheat crop and was attributed to reduced leaf disease and weed infestation (Stevenson and van Kessel, 1996b). Therefore, the remaining 9% of the yield benefit was due to an increase in the N supplying power of the soil. In the present greenhouse study, weed pressure was not a factor and there was no evidence of leaf disease in wheat plants following pea or canola. The yield benefit, and in particular, the non-N benefit of growing wheat

following pea was not as great in the present greenhouse study relative to the landscape-scale field study (Stevenson and van Kessel, 1996b)—although in some studies, the N benefit may be the strongest factor influencing cereal yield increases (Beckie et al., 1997). Nevertheless, non-N benefits including improved micronutrient supply or release of growth promoting compounds can improve the yields of cereal crops following pulses (Stevenson and van Kessel, 1996a). The increase in wheat yield following pea compared to canola may be attributable to differences in soil biology and the presence of symbiotic microorganisms—for example, canola was reported to suppress arbuscular mycorrhizal fungal colonization of flax, thus reducing micronutrient and phosphorus uptake (McGonigle et al., 2011).

6.6.2. Residue N contributions to the subsequent wheat crop

In total, 5.4% of wheat-N was derived from residues of preceding crops—1.8 and 3.6% from AG and BG residues, respectively—while the remaining N was acquired from fertilizer and soil N. This is relatively low considering that %Ndfr from pea ranged between 4.8 and 24.8% when only the aboveground and intact root residues were included (Jensen, 1994a; Senaratne and Hardarson, 1988). Indeed, Mayer et al. (2003a) reported that 18% of wheat- and rape-N was derived from the rhizodeposits, roots, and aboveground straw residues of pea. In the present study, wheat-N derived from the AG and BG residues of pea and canola was 5.0 and 6.4%, respectively. The relatively low %Ndfr in the present study is partially attributable to the addition of N fertilizer. Indeed, the availability of a large source of non-labeled N (i.e., fertilizer in this case) decreases %Ndfr (Hood et al., 1999; Jensen, 1994a). Moreover, the tendency for greater %Ndfr in wheat grown on canola residues is due to the greater total input of N from the residues of canola relative to pea (Table 6.1). Whereas %Ndfr determines the contribution of residue N to total wheat N uptake, examining the recovery of residue N in wheat provides a better comparison of the supply of N from different crop residue sources when they are applied to the soil in differing quantities (Hood et al., 1999).

Decomposition and N mineralization rates differ between residue type and among crop species (Soon and Arshad, 2002). Thus, the supply of N from crop residues to crops may vary similarly. Wheat recovered the highest proportion of residue ^{15}N from the BG residues of pea (13.1%; Fig. 6.3). Recovery of ^{15}N in wheat was within range (8 to 20%) of that reported in previous studies that supplied ^{15}N-labeled pea straw, roots, and rhizodeposits to wheat (Lam et

al., 2012; Mayer et al., 2003a). Reports from other studies using a variety of crops indicate that ^{15}N recovery from above or belowground residues can vary quite widely: 3 to 40% of ^{15}N from the BG residues of lupin was recovered in wheat (McNeill and Fillery, 2008; Russell and Fillery, 1996a); barley recovered 15% of N from pea AG residues (Jensen, 1994a); and wheat recovered 5.5% of N from lentil AG residues (Bremer and van Kessel, 1992a). In the present study, only 7.3% of the N applied in the AG residues of canola was taken up by wheat, which did not differ with the recovery of AG residue ^{15}N from pea, indicating that pea BG residues were most susceptible to mineralization.

The C to N ratio of crop residues is a controlling factor in the N immobilization and mineralization dynamics following incorporation of residues into soil (Nicolardot et al., 2001). The C to N ratio of the AG residues for all crops was wide in this study—148 for canola and 84 for pea (averaged for both N treatments). In contrast, the C to N ratio of the root residues was 79 for canola and 24 for pea. As a result, little mineralization of N from these added AG residues was expected and the contribution of residue-N from pea and canola with lower C to N ratios is likely to be higher than reported here. Moreover, canola roots and shoots have higher acid detergent fiber and lignin concentrations than pea, indicating a more recalcitrant residue (Sangster et al., 2010). However, despite a narrower C to N ratio of pea compared to canola root residues, Soon and Arshad (2002) observed similar N mineralization. Plant residues low in N will release N slowly, supplying little to the subsequent crop although the N is retained in the soil (Handayanto et al., 1997). In the absence of growing plants, residue-derived inorganic N comprised 5% of total inorganic N from oilseed rape residues with a high C to N ratio (72), indicating that some mineralization of the crop residue N had occurred, although this decreased over time (Jensen et al., 1997). Indeed, ^{15}N recovered in the wheat biomass indicates that some organic N contained in the crop residues of the preceding crops was mineralized and accessed by the succeeding wheat crop—or was directly taken up as monomeric N—despite the wide C to N ratios. Detection of ^{15}N in the soil inorganic N pools further supports the mineralization of residue-derived N. Initial immobilization of soil inorganic N was likely to occur with the addition of the residues with wide C to N ratios. In an incubation study, Trinsoutrot et al. (2000) found that the inorganic ^{15}N released as a percentage of residue N increased slowly for canola residues with a high C to N ratio (pods=112; stems=140), but increased more quickly when canola residues with a low C to N ratio were used (pods=33; stems=41). In the present study,

only a small proportion of inorganic N was derived from residues (Fig. 6.5). Therefore, it is likely that the vast majority of residue-N was retained in recalcitrant organic forms (Jensen, 1996b; Trinsoutrot et al., 2000), as microbial residues (Mayer et al., 2003a; Mayer et al., 2004), and immobilized by the soil microbial biomass (Bremer and van Kessel, 1992b; Jensen et al., 1997; Mayer et al., 2003a).

Wheat recovery of ^{15}N from pea may be overestimated, as total recovery from pea BG residues was 107%, though these were similar to values reported elsewhere (98-106%) (Mayer et al., 2003a; Soon and Arshad, 2002). Incomplete recovery of ^{15}N from AG residues may be due to overestimation of the ^{15}N initially applied and/or loss from the system. For example, 5, 15, and 18% of ^{15}N from residues of mustard, potato, and wheat, respectively were lost in a field study (Delgado et al., 2010), indicating that the potential for N loss may vary among the residues of different crop species. McNeill and Fillery (2008) also reported that 50% of lupin BGN was lost from the soil by the time a subsequent mature wheat crop was harvested in a field study. Further investigations that directly measure gaseous ^{15}N-loss are required to determine whether the lower recoveries of ^{15}N from AG residues were due to gaseous loss (pots were lined with plastic, thus leaching was prevented) or cumulative errors in the ^{15}N mass balance.

6.7. Conclusion

The input of N from BG residues was greater than from AG residues, regardless of crop species, and was largely due to a greater contribution of rhizodeposited N compared to N in intact roots. As a result, the percentage of wheat N derived from BG residues was greater than AG residues. This study demonstrates the importance of including BG residue N—including N rhizodeposition—contributions to the total crop residue input. Crop residue supply of N to succeeding wheat was relatively low—contributing between 5.0 and 6.4% of total wheat N—from both pea and canola, respectively, likely due to the relatively low N status of the residues and the addition of inorganic N fertilizer required to support wheat growth. The larger input of N from canola resulted in higher %Ndfr in wheat compared to pea residues; however, when considering the amount of ^{15}N recovered in wheat as a percentage of residue added, a higher amount was recovered from pea BG residues relative to the corresponding pea AG residues and both AG and BG residues of canola. The increased recovery of pea BG residue N in wheat may be attributed to an improved quality of these residues as reflected in the lower C to N ratio of the

pea roots relative to canola, but may also be confounded by potential errors associated with estimating the total BG residue input. Future work should examine potential gaseous losses of ^{15}N from labeled residues of differing types (e.g., rhizodeposits *vs*. straw) and from different crop species. Using direct ^{15}N labeling that includes estimates of rhizodeposition, the contribution of residue-N from both pea and canola to a succeeding wheat crop and soil inorganic N pools was identified despite initially high C to N ratios of the crop residues.

7. INFLUENCE OF FIELD PEA AND CANOLA ON RHIZOSPHERE DENITRIFIER GENE ABUNDANCE AND DENITRIFICATION ENZYME ACTIVITY

7.1. Preface

Plant roots can influence soil nitrogen (N) cycling through the release of carbon (C) and N containing compounds via rhizodeposition. In Chapter 5, I found that both root biomass and N rhizodeposition were greater under canola than field pea. However, a higher proportion of N rhizodeposition was in the soil inorganic N pool in pea compared to canola. Given that there are differences in both the quantity of N and the quality of the compounds released through rhizodeposition between crop species, investigations of the effect of plant roots and rhizodeposits from different crop species on soil N processes is warranted. This chapter examines the influence of pea and canola on denitrification—the abundance of denitrifying genes in the rhizosphere and denitrification enzyme activities in whole soils were determined. In addition, I examined the correlation between N rhizodeposition and measures of denitrification.

7.2. Abstract

Rhizodeposition is the key driver of denitrification associated with plant roots. Previous studies examining the influence of rhizodeposition on denitrification have focused on C since denitrifiers are predominantly heterotrophic. However, N compounds are also released through rhizodeposition, potentially supplying NO_3^- for use as an electron acceptor in denitrification. The effect of mature field pea and canola on the abundance of denitrifying genes in the rhizosphere as well as the denitrification enzyme activity in whole soils (i.e., bulk and rhizosphere soil) was determined in this study. Nitrogen rhizodeposition was estimated based on the root-derived ^{15}N recovered in the rhizosphere and bulk soils of pea and canola supplied with ^{15}N using the cotton-wick technique. Overall, denitrification enzyme activity was relatively low, but differed significantly among crop treatments, with the greatest activities occurring for canola and *Rhizobium*-inoculated pea relative to N-fertilized non-inoculated pea. Abundances of the nitrite reducing gene (*nirS*) and the nitrous oxide reducing gene (*nosZ*) were significantly greater in the

rhizosphere of *Rhizobium*-inoculated pea relative to canola, and *nirS* abundance was greater in inoculated pea relative to N-fertilized non-inoculated pea. There was no crop treatment effect on the nitrite reducing gene, *nirK*. Whereas denitrification enzyme activity did not correlate with gene abundance of any of the genes, there was significant correlation between denitrification enzyme activity and root-derived ^{15}N. The presence of plant roots influenced denitrification enzyme activity and denitrifier gene abundance in different crop treatments to varying degrees, but these effects were uncoupled. Further research employing both ^{13}C and ^{15}N labeling of plants may provide greater insight into the role that rhizodeposition plays in denitrification.

7.3. Introduction

Denitrification is a microbial respiratory pathway involving the stepwise reduction of nitrate (NO_3^-) to dinitrogen gas (N_2). Under limited oxygen or anaerobic conditions, nitrogen oxides are used as terminal electron acceptors resulting in the production of nitrite (NO_2^-), and the gases, nitric oxide (NO) and nitrous oxide (N_2O), as intermediates between NO_3^- and N_2. The release of NO and N_2O can lead to deleterious environmental consequences, as NO contributes to tropospheric pollution and N_2O is a potent greenhouse gas. Denitrification is an important component of the N cycle in agricultural soils, with consequences to the wider ecosystem. In particular, agricultural soils are the greatest single source of N_2O emissions in Canada (Rochette et al., 2008). The conversion of soluble N to gaseous products during denitrification has the negative effect of reducing the N available for plant uptake, but also provides an ecosystem service by diverting potentially harmful N forms, namely, NO_3^- from waterways and N_2O from the atmosphere if N_2O is reduced to N_2 (Philippot et al., 2009a). Examining the factors that influence denitrification is vital to understand the influence that cropping systems may have on the potential for N loss from agricultural soils.

Denitrification rates are influenced by proximal or distal controls (Wallenstein et al., 2006). Proximal controls affect instantaneous denitrification rates and include NO_3^- availability, O_2, pH, and temperature, whereas distal controls are those factors that have a long-term influence on the denitrifying community, and include factors such as soil climate, soil disturbance, and resource availability (Wallenstein et al., 2006). In particular, soil C is strongly linked to denitrification enzyme activity since the majority of denitrifiers are heterotrophic (Coyne, 2008).

Denitrification is carried out by a wide range of facultative anaerobic bacteria, archaea, and eukaryotes (Hallin et al., 2009), each of which may not produce the entire suite of enzymes necessary to carry denitrification to completion (Miller et al., 2008). Examining the functional guilds associated with denitrification provides more meaningful information on the influence of microorganisms on this process, rather than single species approaches (Wallenstein and Vilgalys, 2005). Molecular techniques are used to examine the composition and size of the denitrifying community by targeting the genes that encode denitrification enzymes. The enzymes involved in denitrification are nitrate reductase (nar), nitrite reductase (nir), nitric oxide reductase (nor), and nitrous oxide reductase (nos), which are generally produced under anaerobic conditions only (Eick and Stöhr, 2012).

In an agricultural context, the abundance of various denitrifying genes varies with N fertilization history (Hallin et al., 2009), plant species (Bremer et al., 2007) and crop residue types (Chèneby et al., 2010), and between land use types and history (Dandie et al., 2011; Morales et al., 2010). Differences in community size rather than composition were found to correlate with differences in potential denitrification rates (Hallin et al., 2009). Numerous authors have reported that denitrifier gene abundance correlates with potential denitrification rates and N_2O emissions (Attard et al., 2011; Enwall et al., 2010; Hallin et al., 2009; Morales et al., 2010; Philippot et al., 2009b). However, lack of correlation between denitrifier gene abundance and potential denitrification rates has also been reported (Chèneby et al., 2010; Chèneby et al., 2004; Dandie et al., 2008; Dandie et al., 2011; Henderson et al., 2010; Miller et al., 2008). Moreover, the abundance of one denitrifying gene may correlate with potential denitrification activity, while another denitrifying gene may not (Attard et al., 2011; Hallin et al., 2009). Morales et al. (2010) suggested that the abundance of denitrifying bacterial genes may be used as a proxy for N_2O emissions from soils. Therefore, determining denitrifying gene abundance may be useful in understanding potential for N loss from soils via denitrification.

Plant roots create unique conditions within the rhizosphere that influence factors that regulate denitrification—namely, C availability, soil nitrate levels, and oxygen concentration. The stimulating effect of plant roots on denitrification was first reported over fifty years ago (Woldendorp, 1962). Anaerobic conditions within the rhizosphere can occur due to root and root-associated microbial respiration (Qian et al., 1997), creating microsites for denitrification in soils that are otherwise aerobic. The release of C through rhizodeposition is thought to be the

primary driver of denitrification in the rhizosphere (Philippot et al., 2009a), but the influence of rhizodeposition on measures of denitrification may vary according to the composition of the compounds released from plant roots (Henry et al., 2008). Bremer et al. (2007) suggested that differences in the community composition of *nirK* denitrifying genes among different plant species was due to root exudates. Indeed, the use of different rhizodeposits by specific populations of microorganisms can result in the development of varied microbial communities (Paterson et al., 2007). Therefore, differences in rhizodeposition among crop species may result in variations in microbially mediated soil processes, such as denitrification.

In addition to C rhizodeposition, roots provide a significant input of N to soil (Wichern et al., 2008). Root-derived N may be released to the soil directly as NH_4^+ or NO_3^-, as relatively recalcitrant organic forms, and as labile organic forms that can be easily mineralized and subsequently nitrified to NO_3^-, thus providing NO_3^- for use as an electron acceptor by denitrifiers. Even with an adequate C source, soil NO_3^- availability can limit denitrification. With ample soil NO_3^-, denitrification was higher in the rhizosphere of corn and orchard grass than in unplanted controls; however, at low soil NO_3^-, the reverse was true (Smith and Tiedje, 1979). Similarly, denitrification rates were higher in planted compared to unplanted control soils only at early growth of maize when C rhizodeposition was greatest, and soil NO_3^- was not limiting (Qian et al., 1997). Sharma et al. (2005) suggested that differences in the active denitrifier community profiles between the rhizospheres of pea, lupin and faba bean, may have been due to differences in N rhizodeposition as determined in a related study (Mayer et al., 2003b). Therefore, N rhizodeposition may influence denitrification to varying degrees depending on plant species.

The objectives of this study were to determine the denitrification enzyme activity (DEA) and denitrifier gene abundance in soils of mature pea and canola grown under controlled conditions in a greenhouse, and to determine if there was correlation between N rhizodeposition, DEA, and gene abundance. Plants were labeled with ^{15}N using the cotton-wick method in order to estimate amounts of N released to soil from rhizodeposition. Quantitative PCR (qPCR) was used to determine the abundance of the denitrifier genes, *nirK* and *nirS*, which encode the copper and cytochrome cd_1 nitrite reductases, respectively, and *nosZ*, which encodes nitrous oxide reductase, in the rhizosphere. The nitrite reducers regulate the initial conversion of soluble N (NO_2^-) to a gaseous form (NO), representing a loss of N from the soil system. Nitrous oxide reducers convert the potent greenhouse gas, N_2O, to inert N_2.

7.4. Materials and Methods

7.4.1. Plant growth and soil sampling

Pea and canola plants were each grown in pots containing soil collected from an agricultural research field site and mixed with silica sand in a 1:1 ratio by weight. The resulting soil-sand medium was loam in texture, had a pH of 7.1 (1:2 soil:H_2O), and contained 0.60 g total N kg^{-1}, 6.0 g organic C kg^{-1} soil, 9 mg NO_3^--N kg^{-1}, 29 mg P kg^{-1}, 220 mg K kg^{-1}, and 3.6 mg S kg^{-1}. Soils were packed to a bulk density of 1.4 g cm^{-3} in pots (20 cm dia., 20 cm deep) that were lined with a plastic bag to prevent solution loss via leaching. Plants were grown in a greenhouse and watered to ~80% field capacity (40% water filled pore space) throughout growth and harvested at maturity. In order to estimate N rhizodeposition, pea and canola were supplied with 0.4% (w/v) ^{15}N-urea (99.2 atom% ^{15}N) to the stem of the plant via the cotton-wick method. Details of the ^{15}N-labeling method and N rhizodeposition calculations are described in Chapter 5 (section 5.4). Canola plants received 192 mg N $plant^{-1}$ as urea fertilizer. Nitrogen fertilized canola and non-fertilized pea inoculated with *Rhizobium leguminosarum* were compared since these treatments represent realistic management. An additional N-fertilized, non-inoculated, pea treatment was included to evaluate any differences specifically due to fertilization. At crop maturity, the aboveground components of the plants were harvested and the pots were dismantled to sample the roots and soils. Plant and soil ^{15}N and N data from these harvested samples are presented and discussed in detail in Chapter 5. Subsamples of the rhizosphere and bulk soil were collected immediately following aboveground harvest. The rhizosphere soil—defined as the soil adhered to the root following gentle shaking of the root system—was collected from along the primary and lateral roots using tweezers and stored in 1.5 mL Eppendorf tubes. Visible root fragments were removed from a subsample of soil and this bulk soil was stored in 50 mL Falcon tubes. The subsamples of the rhizosphere and bulk soils were stored at -20°C until analysis.

7.4.2. Soil denitrification enzyme assay and chemical analyses

Denitrification enzyme activity provides an estimate of the indigenous denitrifier population (Drury et al., 2008), and is based on the principle that under non-limiting conditions (i.e., ample NO_3^- and C), the rate of denitrification is proportional to enzyme concentration

(Tiedje, 1994). Acetylene is used in the assay to prevent the reduction of N_2O to N_2. There was insufficient rhizosphere soil to conduct the assay; therefore, DEA was determined on composite samples of bulk and rhizosphere soils. The composite samples were made from subsamples of the bulk and rhizosphere soils, which were combined in proportions equal to the proportions of total rhizosphere to total bulk soil of the whole pot (i.e., subsampled soils plus soils recovered after complete root sampling as described in Chapter 5). The soils were thawed and stored at 4°C prior to the assay. Soils were combined in 125 mL glass bottles to achieve a total of 5 g dry-weight equivalent moist soil. Each soil sample received 10 mL of solution containing 7.12 m*M* KNO_3, 2.78 m*M* glucose, and 1 g L^{-1} chloramphenicol. Each sample was assayed in duplicate. The bottles were capped with a rubber septum and metal crimping cap, flushed with ultra high purity N_2 gas to remove O_2, and vented to atmospheric pressure with a needle. The assay began with a one-time injection of 10 mL acetylene. Following mixing with a syringe for 15 s, a 10 mL sample was taken from the bottle and stored in a pre-evacuated 12.0 mL Exetainer® vial (Labco Ltd., UK). To replace the sample volume, 10 mL of N_2 were injected into the bottle. Following this initial sampling, the bottles containing the soil-slurry were shaken at 125 rpm on a rotary shaker. Gas samples were then taken 30, 60, and 90 min after the initial acetylene injection in the same manner as the first sampling. Soil-slurry samples were returned to the rotary shaker between each sampling period. The gas samples were analyzed for N_2O concentration using gas chromatography (Bruker 450-GC, Bruker Chemical Analysis BV, Goes, NL). The amount of N_2O ($\mu g\ kg^{-1}$ soil) at each interval was calculated and the slope of the linear regression was used to determine the production rate of N_2O ($\mu g\ N\ kg^{-1}\ soil\ h^{-1}$) (Drury et al., 2008).

Soil inorganic N was extracted from composite bulk and rhizosphere soil samples using 2 *M* KCl and the extracts analyzed for NO_3^- plus NO_2^- and NH_4^+ on a SmartChem™ 200 (Westco Scientific Instruments, Brookfield, CT); these data are presented in Chapter 5 (Table 5.4). Dissolved organic C (DOC) was determined on composite bulk and rhizosphere soil samples according to the water-extractable organic matter method described in Chantigny et al. (2008). Extracts were analyzed for C on a TOC-V total organic C analyzer (Shimadzu Corporation, Kyoto, Japan).

7.4.3. Quantifying denitrifier gene abundance

Total DNA was extracted from rhizosphere soils of pea and canola using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA was quantified (ng μL^{-1}) using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at -20ºC until analysis by qPCR.

Quantitative PCR was used to estimate the abundance of the nitrite reducing genes, *nirK* and *nirS*, and the nitrous oxide reducing gene, *nosZ*, in four replicate soils of each crop treatment. Standards for *nosZ* were prepared using pre-extracted genomic DNA of *Pseudomonas stutzeri* (ATCC 14405). Standards for *nirK* and *nirS* were made from a linearized plasmid containing the synthesized gene fragment sequenced from *Sinorhizobium meliloti* 1021 and *Pseudomonas stutzeri* (ATCC 14405), respectively (Biomatik, Cambridge, ON). The primers used and the thermocycling conditions are highlighted in Table 7.1. For each gene, at least five 10^{-1} dilutions of the standard were used to construct the standard curve used to quantify gene copy number. An additional set of soil DNA samples was spiked with a known amount of standard DNA to determine whether the former contained compounds that inhibited the qPCR. All reactions were carried out in triplicate. No template controls were included in each assay. Efficiencies of the qPCR were determined based on the slope of the standard curve (r^2=0.99) and were 80%, 88%, and 85% for *nirS*, *nosZ*, and *nirK*, respectively. Quantitative PCR was performed for *nosZ* on an ABI 7500 Real-Time PCR System, while *nirK* and *nirS* were determined on a StepOnePlus™ Real-Time PCR System (both qPCR machines manufactured by Applied Biosystems, Foster City, CA). Reactions were performed in a total volume of 25 µL and consisted of the following: 2 µL template DNA; 12.5 µL QuantiTect SybrGreen PCR Master Mix (Qiagen, Toronto, ON); 1 µL (*nirK*) or 2.5 µL (*nirS* and *nosZ*) forward and reverse primers; 0.125 µL bovine serum alba (*nirS* and *nirK*); and the remaining volume consisted of nuclease-free deionized water. Specificity of the reactions was confirmed by a single peak in the melt curve analysis and the presence of a single band of the expected size for each gene after electrophoresis on a 1% agarose gel.

Table 7.1. Targeted genes, primers, and thermocycling conditions used in the quantitative PCR of extracted DNA from the rhizosphere soils of canola, N-fertilized pea, and non-fertilized pea that were grown under greenhouse conditions and labeled with ^{15}N-urea using the cotton-wick method.

Gene target	Primer set	Primer concentration (μ*M*)	Sequence (5' – 3')	Amplicon length (bp)	Thermocycling conditions	Reference
nirS†	nirSCd3aFm nirSR3cdm	1.0	AAC GYS AAG GAR ACS GG GAS TTC GGR TGS GTC TTS AYG AA	365	1 cycle: 95°C, 15 min 6 cycles: 95°C for 15 s, 63 to 58°C for 45 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s 40 cycles: 95°C for 15 s, 60°C for 45 s, 72°C for 30 s, 80°C for 15 s 1 cycle: 95°C for 15 s, 60 for 1 min, 95°C for 15 s	Kandeler et al. (2006)
nirK‡	nirK876 nirK1040	0.5	ATY GGC GGV CAY GGC GA GCC TCG ATC AGR TTR TGG TT	165	1 cycle: 95°C, 15 min 6 cycles: 95°C for 15 s, 63 to 58°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s 40 cycles: 95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s 1 cycle: 95°C for 15 s, 60 for 1 min, 95°C for 15 s	Henry et al. (2004)
nosZ§	nosZ2F nosZ2R	1.0	CGC RAC GGC AAS AAG GTS MSS GT CAK RTG CAK SGC RTG GCA GAA	267	1 cycle: 95°C, 15 min 40 cycles: 95°C for 15 s, 57°C for 30 s, 72°C for 30 s, 80°C for 35 s 1 cycle: 95°C for 15 s, 60 for 1 min, 95°C for 15 s	Henry et al. (2006)

† Standard curves made from linearized plasmid containing synthesized *nirS* gene fragment sequenced from *Pseudomonas stutzeri* (ATCC 14405)
‡ Standard curves made from linearized plasmid containing synthesized *nirK* gene fragment sequenced from *Sinorhizobium meliloti* 1021
§ Standard curves made from genomic DNA of *Pseudomonas stutzeri* (ATCC 14405)

7.4.4. Statistics

One-way analysis of variance was performed with crop treatment as a fixed effect and block as a random effect. Gene copy numbers were log transformed for the analysis of variance. Normality of residuals was tested using the Shapiro-Wilk statistic and homogeneity of variances was tested using Levene's test. Means comparisons were made using Tukey's Honestly Significant Difference test. Pearson's correlation coefficients were calculated for the relationships between N rhizodeposition parameters, soil inorganic N, DOC, enzyme activity, and gene abundance. All tests were declared significant at $P \leq 0.05$. Statistical analyses were performed using SPSS® Statistics version 20.0 for Mac (IBM Corp., 2011).

7.5. Results

7.5.1. Denitrification enzyme activity and dissolved organic carbon

Denitrification enzyme activity in the combined bulk and rhizosphere soil samples differed among crop treatments ($P<0.001$), with the greatest activity occurring in soils of canola and inoculated pea that had not received N fertilizer (Fig. 7.1). Enzyme activity in soils of non-fertilized inoculated pea was 45% higher than in soils of N-fertilized pea. In contrast, DOC did not differ among crop treatments (Fig. 7.2; $P=0.728$). Averaged across all treatments, DOC was 6.8 mg kg^{-1} soil.

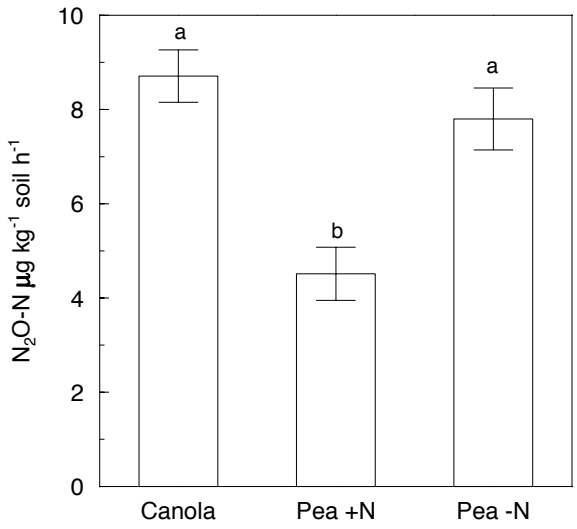

Fig. 7.1. Potential denitrification enzyme activity in bulk and rhizosphere composite soil samples of mature canola, pea N-fertilized (+N), and pea non-fertilized (-N) grown in a greenhouse. Same letters above bars ± standard error of the mean (n=4) indicate no significant difference between treatments according to Tukey's HSD test (*P*>0.05).

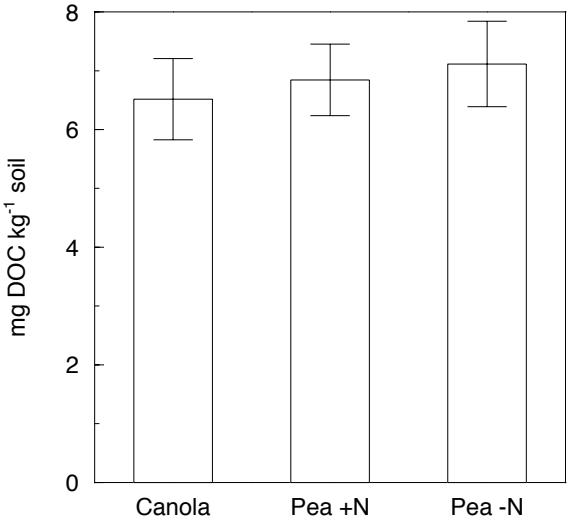

Fig. 7.2. Dissolved organic carbon in bulk and rhizosphere composite soil samples of mature canola, pea N-fertilized (+N), and pea non-fertilized (-N) grown in a greenhouse. Bars indicate standard error of the mean (n=4).

7.5.2. Denitrifying bacteria gene abundance

Quantitative PCR was used to determine the abundance of denitrifying genes in the rhizospheres of canola, inoculated pea, and N-fertilized non-inoculated pea. There was no difference in the abundance of *nirK* genes within the rhizosphere of the three crop treatments (P=0.718). However, crop treatment significantly affected the abundance of *nirS* (P<0.001) and *nosZ* (P=0.016). The abundance of *nosZ* gene copies in the rhizosphere of non-fertilized, inoculated, pea was greater than canola, while *nirS* abundance was greatest in the rhizosphere of non-fertilized, inoculated, pea compared to both other crop treatments (Fig. 7.3). Whereas the abundance of *nirS* was less than that of *nirK* and *nosZ* across all treatments (paired sample t-test; P<0.001), there was no difference in abundance between *nirK* and *nosZ* (P=0.273).

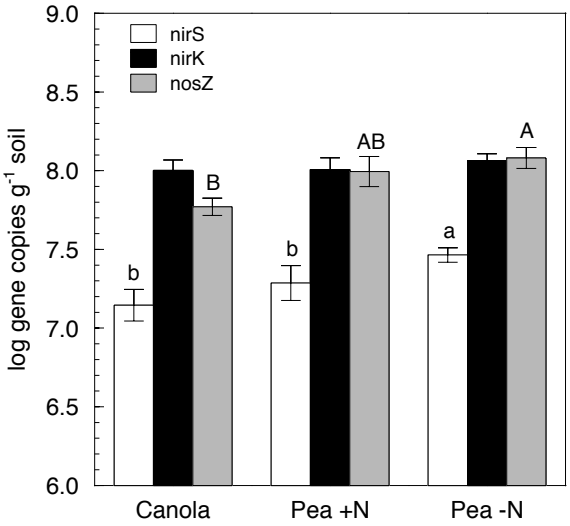

Fig. 7.3. Abundance of denitrifying genes (log gene copies g^{-1} soil), *nirS*, *nirK*, and *nosZ*, in the rhizosphere soils of mature canola, pea N-fertilized (+N), and pea non-fertilized (-N) grown in a greenhouse. Same lower- or upper-case letters above bars with standard errors (n=4) indicate no significant difference among crop treatments within a gene according to Tukey's HSD test (P>0.05).

7.5.3. Correlation between rhizodeposition and denitrification parameters

Denitrification enzyme activity was significantly correlated to ^{15}N in the bulk soil and tended to correlate with root biomass (P=0.072), but was not related to amounts of root N or rhizodeposited N (Table 7.2). Similarly, there was no correlation between the abundance of denitrifying bacterial gene copies and measures of rhizodeposition or root parameters with denitrification enzyme activity. There was significant correlation between the abundance of *nosZ* and *nirS* gene copies.

Table 7.2. Pearson correlation coefficients (n=12) for plant root biomass and N, bulk soil (BS) and rhizosphere soil (RS) ^{15}N enrichment, N derived from rhizodeposition (NdfR), soil nitrate and dissolved organic C (DOC), denitrification enzyme activity (DEA), and rhizosphere gene abundance data of mature canola, N-fertilized pea, non-fertilized pea grown in the greenhouse and supplied with ^{15}N using the cotton-wick ^{15}N labeling technique.

	Root mass	**Root N**	**BS ^{15}N**	**RS ^{15}N**	**$NdfR_{BS}$**	**$NdfR_{RS}$**	**NO_3^--N**	**DOC**	**DEA-N_2O**	***nirK***	***nirS***	***nosZ***
	g plant^{-1}	*mg plant^{-1}*	*mg ^{15}N plant^{-1}*		*mg plant^{-1}*				*μg N kg^{-1} h^{-1}*	*log gene copies g soil^{-1}*		
Root mass	1	0.95**	0.88**	0.89**	0.85**	0.89**	-0.84**	-0.01	0.54	-0.14	-0.34	-0.44
Root N		1	0.80**	0.78**	0.74*	0.82**	-0.92**	0.24	0.42	-0.17	-0.31	-0.46
BS ^{15}N			1	0.91**	0.87**	0.90**	-0.73*	0.16	0.60*	-0.17	-0.44	-0.54
RS ^{15}N				1	0.73*	0.88**	-0.73*	-0.23	0.51	-0.14	-0.35	-0.29
$NdfR_{BS}$					1	0.92**	-0.75**	0.38	0.50	-0.10	-0.45	-0.46
$NdfR_{RS}$						1	-0.85**	-0.01	0.41	-0.14	-0.26	-0.23
NO_3^--N							1	-0.25	-0.30	0.38	0.33	0.38
NH_4^+-N								1	-0.29	-0.37	-0.48	-0.56
DEA-N_2O									1	-0.12	-0.17	-0.34
nirK										1	0.35	0.51
nirS											1	0.72**
nosZ												1

*,**Significantly correlated at $P \leq 0.05$ and 0.01, respectively

7.6. Discussion

Differences in root biomass and N rhizodeposition among canola and pea were reported in Chapter 5 and, in this complementary study, it was hypothesized that the presence of roots from these two crop species would influence denitrification activity and abundance of denitifier genes to varying degrees. Denitrification enzyme activity did not differ in soils of canola and inoculated pea; however, DEA was 45% lower in soils of N-fertilized pea. It was expected that DEA would be greater in the N-fertilized pea and canola treatments relative to the inoculated non-fertilized pea due to the addition of urea fertilizer to the former crop treatments. However, by the time the plants reached maturity, soil inorganic N was similar between the two pea treatments (34.9 *vs*. 31.8 mg plant^{-1}), whereas inorganic N was significantly lower under canola (20.5 mg plant^{-1}) likely due to greater N demand by canola over the course of growth, as presented and discussed in Chapter 5. Therefore, the observed differences in DEA among the crop species were independent from the soil inorganic N levels at the end of the growth period. Moreover, there was no correlation between inorganic N and DEA. Despite lower inorganic N under canola, DEA was higher than the N-fertilized pea, and despite similar inorganic N between pea treatments, DEA was higher for the non-fertilized pea. However, there was significant correlation between ^{15}N in the bulk soil and DEA.

Overall, DEA ranged between 4.5 and 8.7 μg N_2O-N kg^{-1} soil h^{-1}, which is low relative to studies reviewed by Coyne (2008). For example, daily addition of artificial root exudates to soils over one month period resulted in DEA of 560 μg N_2O-N kg^{-1} soil h^{-1} (Henry et al., 2008), similar to that determined in soil amended with crop residue or glucose (500 to 1400 μg N_2O-N kg^{-1} soil h^{-1}) , alone or in combination with KNO_3 in an incubation study (Miller et al., 2008). In my study, the plants were grown under unsaturated soil conditions, which would result in lower denitrification enzyme activity than if the soils were maintained at higher moisture. Nevertheless, denitrification enzyme activity can persist even after significant periods of air drying (Freney et al., 1979; Smith and Parsons, 1985). In addition, sample storage at -20ºC and at 4ºC likely depressed denitrification enzyme activity (Luo et al., 1996; Tiedje, 1982). Due to the relatively small rhizosphere sample size, DEA activity was determined on a composite sample containing both rhizosphere and bulk soil in proportions representing the whole pot, but was predominantly comprised of bulk soil—as a result, the DEA may have been higher within the much smaller volume of soil surrounding the root.

Carbon availability is one of the key drivers of denitrification activity, as the majority of denitrifiers are heterotrophic. Canola produced more root biomass and had a higher C to N ratio than pea (Chapter 5), and therefore contributed more root-C to the soil as root detritus and possibly more in root exudates compared to pea. Moreover, although not significant, root biomass tended to correlate with DEA (P=0.072), and ^{15}N in the bulk soil significantly correlated with DEA (Table 7.2). In a ^{13}C pulse labeling study, estimates of belowground C (i.e., from roots and rhizodeposition) from canola was greater than pea (Sangster et al., 2010). The higher belowground C of canola compared to pea was confirmed in a follow-up study, which also reported that more root-derived C g^{-1} SOC was recovered in the water extractable organic matter pool in pea compared to canola (Comeau, 2012). The greater contribution of root-derived C to more soluble soil C pools under pea may be due to the higher concentration of recalcitrant compounds in canola relative to pea roots (Sangster et al., 2010). Moreover, in the present study the C to N ratio of canola roots was particularly high (79) relative to pea (24); therefore, canola roots were not likely to decompose very quickly. As a result, while there was likely a greater input of rhizodeposited C from canola roots, the release of root-derived C into the soluble pool as a proportion of total root-derived C was likely low from canola compared to pea. The total amount of root-derived C in the soluble pool may have been similar, or even higher in pea compared to canola. However, this could not be determined without ^{13}C labeling. There was no significant effect of crop treatment on DOC (Fig. 7.2; P=0.728), which averaged 6.8 mg kg^{-1} soil. The differences in DEA among crop treatments, although significant, were very small, which may explain why there was no clear correlation between DOC and DEA in my study. Moreover, the total organic C in these soils was relatively low (6 g kg^{-1} soil), likely contributing to the low denitrification activity overall (Bremner and Shaw, 1958). The input of root-derived C, which was the only input of C that differed among crop treatments, was therefore too small or recalcitrant to increase DOC in the bulk soil to substantially affect denitrification activity.

Higher N_2O emissions and denitrification rates are sometimes observed during growth of legumes relative to non-fixing plants, although this phenomenon is more likely due to the decay of N-rich root and nodule residues rather than the biological N fixation process itself (Rochette and Janzen, 2005). Indeed, DEA was higher in the soils of the inoculated pea compared to the N-fertilized pea. Inaba et al. (2009) reported increased N_2O emission from the rhizosphere soil of late-stage nodulated compared to non-nodulated soybeans, with decaying nodules contributing

more N_2O than fresh nodules. However, there were no differences between N_2O emissions from nodulated and non-nodulated pea or lentil plants grown in sterile Leonard jars or in soil (Zhong et al., 2009). A number of rhizobial species and strains have been shown to possess denitrifying capabilities (Garcia-Plazaola et al., 1993; O'Hara and Daniel, 1985; Zablotowicz et al., 1978). However, strains of *Rhizobium leguminosarum*, which forms the symbiosis with a number of crop species, including pea and lentil, did not reduce nitrate at all (Zablotowicz et al., 1978) or only to a small degree (Daniel et al., 1982). Commercially produced isolates of *R. leguminosarum* bv. *viciae* inoculant did not use NO_3^- as an electron acceptor under anaerobic conditions (Zhong et al., 2009). Therefore, it was not expected that the *R. leguminosarum* inoculant added to the non-fertilized pea treatment would influence denitrification activities directly. However, the presence of rhizobia or active N fixation in legumes may result in conditions that modify the microbial communities, with potential to affect denitrifying bacteria.

The abundance of nitrite reducing bacterial genes *nirS* and *nirK* ranged between 7.1 to 7.5 and 8.0 to 8.1 log gene copies g^{-1} dry soil, respectively, similar to that in soils from various N fertility treatments in a long-term field trial (Hallin et al., 2009). The abundance of the nitrous oxide reducing genes *nosZ* ranged between 7.8 and 8.1 log gene copies g^{-1} dry soil, which was within the range reported in other agriculturally focused studies (Dandie et al., 2011; Hallin et al., 2009; Henderson et al., 2010). The abundance of *nirS* genes was higher in the soils of pea inoculated with *Rhizobium leguminosarum* relative to the non-inoculated N-fertilized pea, but there was no difference in the abundance of *nirK* or *nosZ*. Similarly, Babić et al. (2008) reported no significant difference in the abundance of *nirK* or *nosZ* in the rhizosphere of alfalfa that was inoculated with two different strains of *Sinorhizobium meliloti* compared to the non-inoculated control for all growth stages (early trifoliate stage, bud stage, and late flowering). However, one of the strains increased the abundance of *nirS* in the rhizosphere of late flowering alfalfa relative to the control. Using culture techniques, denitrifying bacterial populations were larger under inoculated pea and lentil plants compared to non-inoculated and non-nodulated plants (Zhong et al., 2009). Therefore, inoculation can influence denitrifying populations in the rhizosphere, although the effect on specific functional genes is inconsistent. In my study, urea fertilizer may have influenced the lower gene abundance in the non-inoculated pea treatment; however, inorganic N was similar between the two pea treatments at the time of sampling (data presented in Chapter 5, Table 5.4).

Denitrification enzyme activity was similar between canola and inoculated, non-fertilized pea, but abundances of two out of the three denitrifying genes were lower in the former crop treatment. The denitrification enzyme activity assay did not separate the production of N_2O from N_2; therefore, there may be differences in the activities of the nitrite reducing versus the nitrous oxide reducing communities. Indeed, the community composition of denitrifiers, which has been found to vary among the rhizospheres of different plant species (Sharma et al., 2005), may influence whether N_2O or N_2 is the dominant end product of denitrification (Hallin et al., 2009). For instance, emissions of N_2O were lower in the rhizosphere of soybean compared to corn suggesting the conversion of N_2O to N_2 in the N-fixing compared to the non-fixing crop (Sey et al., 2010). Further study is necessary to determine if this is the case between canola and pea and to understand the link between denitrifier gene abundance and the relative production of N_2O and N_2. Furthermore, the abundance of transcripts rather than gene copies may provide more useful information on the relationship between the denitrifying community and denitrification, particularly if examining the effects of short-term changes (Morales et al., 2010). Determining the abundance of transcripts provides a measure of the activity and not just the size of the microbial community. However, determining transcript abundance in environmental samples such as soil is challenging due to difficulties in extracting RNA (Smith and Osborn, 2009). Although more recent studies are examining transcript as well as gene abundance in soils (Dandie et al., 2011; Henderson et al., 2010), gene abundance continues to be used to examine denitrification activity and denitrifying communities (Grigulis et al., 2013; Long et al., 2013). As techniques improve, however, the evaluation of transcripts will play a larger and important role in furthering our understanding of the influence of agricultural management and cropping systems on soil microbial ecology and ecosystem function, particularly N cycling.

7.6. Conclusion

Denitrification enzyme activity and abundance of denitrifying genes differed among the crop treatments tested in this study. Whereas DEA was highest under non-fertilized pea and canola, and was lowest under fertilized pea, denitrification gene abundance was highest under non-fertilized pea, but lowest under canola, indicating an uncoupling between enzyme activity and gene abundance. The differences among crop treatments were determined within a single growing season. Whether stronger differences in DEA and denitrifier gene abundance develop

over time between crop rotations that include pulse versus oilseed crops requires further investigation. The low denitrification enzyme activity under N fertilized pea compared to the inoculated pea suggests that the presence of the inoculant, rather than differences in soil inorganic N, influenced denitrification since inorganic N was similar at harvest. There was no correlation between N rhizodeposition and DEA or denitrifier gene abundance, but a tendency for root biomass to correlate with DEA. Since denitrification is strongly influenced by C availability, further research using dual labeling of plants with ^{13}C and ^{15}N and direct determination of $^{15}N_2O$ and $^{15}N_2$ may provide a more complete picture of the influence of rhizodeposition on denitrification.

8. SYNTHESIS AND CONCLUSIONS

The release of compounds into the soil by plant roots has been recognized for over a century (Nguyen, 2003). This input of primarily organic material through rhizodeposition is the principle driver of the formation of the so-called rhizosphere effect (Jones et al., 2009). Much of the initial research that identified compounds released from plant roots was conducted in solution culture rather than in soil (Nguyen, 2003). However, the type and quantity of compounds released from plant roots grown in soil differ from those grown in solution culture (Jones et al., 2009). Methods for labeling plants with ^{14}C and ^{13}C were developed to quantify and identify compounds released from plant roots grown in soil. Whereas the vast majority of research on rhizodeposition has focused on C (Jones et al., 2009), recent developments in ^{15}N-labeling techniques reveal that N released from plant roots comprise a significant component of the total amount of N assimilated by plants throughout development, but the research has been relatively limited (Wichern et al., 2008). The research presented in this dissertation addresses some of the knowledge gaps on the amounts and fate of belowground N released from different field crop species.

The general goal of this research was to quantify the input of N to soil via rhizodeposition from the roots of field pea and canola—important crops grown in rotation with wheat on the Canadian prairies—and to examine the contribution of belowground N to soil inorganic N pools, plant N uptake, and the influence of belowground N on processes of the N cycle (e.g., mineralization and denitrification). Nitrogen-15 labeling techniques were used to trace the plant-derived N into the soil.

8.1 Summary of Findings

Field pea is the major pulse crop grown across the Canadian prairies and its inclusion in crop rotations is vital to improve N management in this region. This is the first study to directly examine the release of biologically fixed N to soil from field pea. As determined using continuous $^{15}N_2$ labeling in a greenhouse study (Chapter 3), the greatest amounts of fixed N were

released during the pod-filling stage (maximum Ndfa in the rhizosphere was 1.45%). Fixation was low and variable within the atmospheric labeling experiment (maximum Ndfa in the whole plant was 18%) likely due to suppression of N fixation by high soil inorganic N levels. Even at these low N fixation rates, the continuous supply of $^{15}N_2$ allowed for the detection of N rhizodeposition. Nitrogen derived from rhizodeposition (NdfR) was calculated based on the ratio of atom% ^{15}N excess in the soil and that in the roots using the Janzen and Bruinsma (1989) equation. Average values in the rhizosphere were 3.6, 2.8, 3.0, and 3.2% for the vegetative, flowering, pod filling, and mature growth stages, respectively. Values were 8.8, 5.8, and 4.2% for the vegetative, flowering, and mature stages when pea grown in the same soil was artificially supplied with ^{15}N urea to the stem using the cotton-wick method (Chapter 4).

When the data were combined from all of the studies in which pea was ^{15}N-labeled and harvested at maturity ($^{15}N_2$ atmospheric method as in Chapter 3, cotton-wick method as in Chapters 4 and 5), there was a significant linear relationship between rhizosphere soil and root ^{15}N enrichment ($P<0.001$; Fig. 8.1), with values from the atmospheric labeling experiment on the lower end of the scales. A positive linear relationship between ^{15}N enrichment of roots and rhizosphere soil of $^{15}N_2$-labeled pea plants across all growth stages was found ($r^2=0.64$, $P<0.001$, Chapter 3), and also occurred for mature pea plants labeled with ^{15}N-urea ($r^2=0.37$, $P=0.080$, Chapter 4; $r^2=0.70$, $P<0.001$, Chapter 5); although comparing the slopes and y-intercepts of these relationships is difficult because the number of $^{15}N_2$ labeled plant and root samples are few and the values are so much lower than for the ^{15}N-urea labeled plants. Using a higher number of plant replicates in the $^{15}N_2$ labeling system and ensuring relatively high and uniform rates of N fixation would allow for easier comparison with results obtained using the cotton-wick ^{15}N-labeling method. However, establishing and maintaining the atmospheric conditions in the $^{15}N_2$-labeling apparatus was a significant technical challenge in this study and in studies by other researchers using different plants (McNeill et al., 1994; Mohr et al., 1998; Russelle et al., 1994; Verburg et al., 2004). Therefore, the cotton-wick method is a practical alternative to atmospheric $^{15}N_2$ labeling for calculating estimates of N rhizodeposition in legumes. Where the goal is to assess the proportion of symbiotically fixed N to total N rhizodeposition, estimates of N fixation and rhizodeposition using ^{15}N isotope dilution and shoot-labeling methods (e.g., cotton-wick technique), respectively, can be made simultaneously under similar growth conditions.

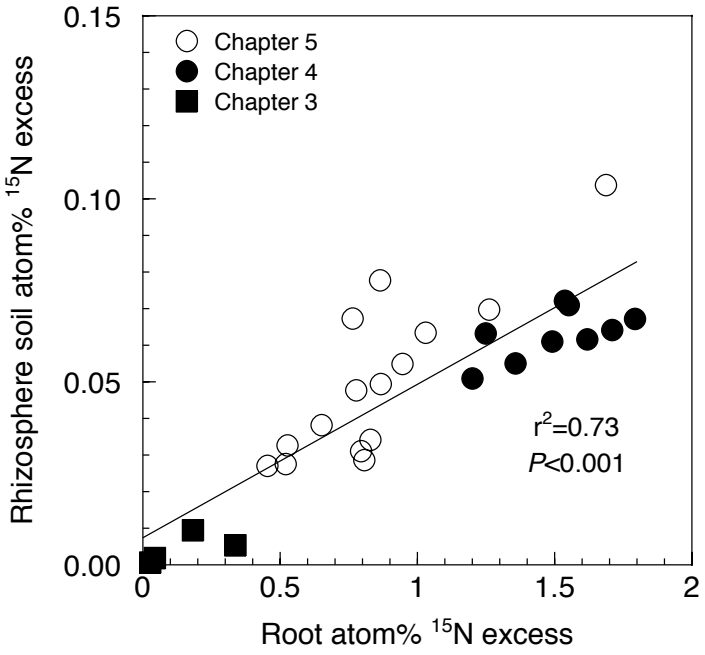

Fig. 8.1. Rhizosphere soil versus root atom% ^{15}N of mature field pea (cv. CDC Meadow) grown under controlled conditions in a greenhouse. Solid filled squares and circles from pea plants grown in soil collected from Scott, SK (Chapter 3 and 4), while white filled circles indicate pea grown in soil collected from Swift Current, SK (Chapter 5). Circles indicate plants continuously supplied with ^{15}N-urea (99.2 atom% ^{15}N excess) to the stem of the plant via a cotton-wick (Chapter 4 and 5), while squares indicate plants grown in conditions with the roots exposed to a continuous supply of $^{15}N_2$ enriched gas (3.0634 atom% ^{15}N excess; Chapter 3)

One of the objectives of this research was to determine the contribution of N rhizodeposition to the total plant N balance. In doing so, the total input of N into soil from all crop residue pools (i.e., straw, roots, and rhizodeposits) can be assessed. Nitrogen rhizodeposition by mature field pea comprised 7.6% and 17.4% of total N in plants labeled with ^{15}N-urea as presented in Chapters 4 and 5, respectively. These results are within range of previous reports of N rhizodeposition in pea using the cotton-wick method. The difference in estimates may be due to differences in the characteristics of the soils used in the two experiments—a Dark Brown Chernozem collected from Scott, SK was used in Chapter 4, while a Brown Chernozem collected from Swift Current, SK, was used in Chapter 5. Slight differences in ^{15}N-labeling frequency as well as differences in growth conditions may also have contributed. Moreover, the NHI was higher in pea plants grown in the study presented in Chapter 4,

indicating that more N was allocated towards seed and foliar development rather than root growth.

Rhizodeposition comprises multiple mechanisms of N release from plant roots, including, but not limited to, root exudation, cell lysis, and root senescence. By using ^{15}N-labeling and by separating rhizosphere from bulk soils, changes in the pattern of N rhizodeposition with time were detected—at early stages of crop growth, the higher atom% ^{15}N excess values in rhizosphere soils suggest that N associated with root exudates dominated N rhizodeposition, while at later stages, N associated with root turnover and unrecovered root fragments dominated N rhizodeposition as suggested by the increase in atom% ^{15}N excess in bulk soils with plant age (Chapter 4). This supports results from previous C and solution culture studies that show a change in quality of rhizodeposition with development (Gransee and Wittenmayer, 2000; Nguyen, 2003; Rovira, 1956). Moreover, Jensen (1996c) found that N rhizodeposits became more recalcitrant with plant age based on incubation of soils containing the ^{15}N-labeled rhizodeposits of pea and barley. The amount of N rhizodeposition increased with plant age, but the proportion of plant N released belowground decreased with time as plants allocated resources to reproductive organs.

Mechanisms of N release and the amounts of N in rhizodeposits differ among crop species. Differences in rhizosphere and bulk soil atom% ^{15}N between canola and pea suggest different modes of N rhizodeposition (Chapter 5). The majority of N rhizodeposition of canola was attributed to unrecovered fine roots, as indicated by higher atom% ^{15}N in the bulk soil relative to the rhizosphere soil. In contrast, the atom% ^{15}N in rhizosphere versus bulk soils in pea was more pronounced, indicating that N associated with root exudates contributed to N rhizodeposition processes to a greater degree for pea than canola. A higher amount of N was released via rhizodeposition by canola compared to pea (59.7 *vs*. 23.8 mg N plant^{-1}), which was related to higher root biomass (r^2=0.60). Previous studies focused on legumes or cereals; this is the first study to quantify N rhizodeposition in canola or any other *Brassica* species. Using cotton-wick ^{15}N labeling, belowground N (roots and rhizodeposits) of canola was determined to comprise 70% of total residue N, while this was only 61% in pea. However, a higher proportion of pea N rhizodeposits contributed to soil inorganic N pools, highlighting a difference in quality of rhizodeposition between the two crops.

Few studies have examined the fate of belowground crop residue N in soils and to succeeding crops (Gardner and Drinkwater, 2009). By using a cross-^{15}N labeling approach with above and belowground residues, it was possible to differentiate the relative contribution of crop residue-N sources to the growth of a succeeding crop (Chapter 6). Belowground residues, including rhizodeposits, of both pea and canola contributed almost twice as much N to the succeeding wheat crop than their corresponding straw residues, highlighting the importance of including estimates of root and rhizodeposition in determining the source and fate of N in cropping systems. Much of this residue-derived N may be cycled internally, but the quality of the residues affect the rate of N mineralization and thus the availability of N to succeeding crops, as well as the susceptibility of N to losses from agricultural soils due to leaching or conversion to gaseous forms. Crop residues can have a relatively low N content since much of the N is allocated towards seed development; therefore, the supply of N to succeeding crops may be small (Lupwayi and Kennedy, 2007). Indeed, the wide C to N ratios of both pea and canola residues resulted in relatively small N contributions to wheat (5.4%). On a per plant basis, canola contributed a higher quantity of N to wheat due to the greater total input of N from canola residues compared to pea, as a result of a higher residue biomass. However, pea residues supplied a higher proportion of N to wheat than canola, indicating that pea residues—particularly roots and rhizodeposits—were more susceptible to mineralization than canola residues.

Rhizodeposition can structure plant-associated microbial communities by providing readily available substrate to the rhizosphere (Berg and Smalla, 2009; Paterson et al., 2007). Furthermore, these associations are crop specific (Garland, 1996). Denitrifying bacterial communities differ among plant species, and it was speculated that these differences were driven by plant species-specific differences in rhizodeposition patterns (Bremer et al., 2007; Sharma et al., 2005). In my study, denitrifier gene abundance differed between pea and canola, with higher abundance of *nirS* and *nosZ* in the rhizosphere of inoculated, non-fertilized, pea. Gene abundance was not related to measures of N rhizodeposition suggesting the difference between crop species may be attributable to other plant factors. Henry et al. (2008) reported no effect of artificial root exudates on denitrifier gene abundance. However, soluble root exudates play a minor role in influencing bacterial communities compared to insoluble rhizodeposits, such as mucilage or sloughed root cells (Dennis et al., 2010). Indeed, C rhizodeposition may be driving the difference between denitrifier gene abundance between the two crop species. Rhizosphere

denitrifying gene abundance was not correlated to denitrifying enzyme activity in the combined bulk and rhizosphere soil sample; however, DEA was correlated with root-derived ^{15}N in the bulk soil and weakly correlated to root biomass (P=0.072), suggesting that the input of fine roots to the bulk soil of canola may have been driving the DEA. Further research is required to determine whether greater input of root fragments to the bulk soil in the case of canola may explain the lack of correlation between gene abundance in the rhizosphere and activity in the bulk soil—although abundance of transcripts rather than genes would provide more useful information on the activity of the denitrifying community.

8.2 Future Research

Future investigations of rhizodeposition should make use of dual ^{15}N and ^{13}C isotope labeling to simultaneously examine the input of N and C into soil and their fate in agroecosystems. Using ^{13}C pulse labeling in a growth chamber, Sangster et al. (2010) determined C partitioning coefficients of grain:straw:root + rhizodeposition to be 0.16:0.51:0.32 for canola and 0.43:0.36:0.21 for pea. The corresponding N partitioning coefficients as determined in my study were 0.44:0.17:0.39 for canola and 0.64:0.15:0.21 for pea. Whereas the partitioning of C and N to roots and rhizodeposits are similar, there are marked differences in aboveground partitioning, highlighting the need to combine ^{13}C and ^{15}N labeling in the same plant under similar growing conditions. Nitrogen and C cycles are inextricably linked—plant N content is related to photosynthesis and the C to N ratio of plant residues influences rates of decomposition (Frank and Groffman, 2009). Therefore, dual labeling provides more powerful information on the fate of rhizodeposits and crop residues and their influence on C and N cycles compared to single isotope labeling.

Root to shoot ratios differ between plants grown in the field and those grown in pots (Poorter et al., 2012). Studying rhizodeposition in the field is therefore warranted, particularly because rhizodeposition is often related to root biomass. A handful of studies have used shoot ^{15}N-labeling methods in cores or microplots to determine N rhizodeposition in the field (López-Bellido et al., 2011; Mahieu et al., 2007; McNeill and Fillery, 2008; Wichern et al., 2007a; Wichern et al., 2007b). However, frequent monitoring and maintenance of the labeling system is required, which limits field studies to sites that are relatively close—an option that is not often available in the Canadian prairies where distances between field sites and research facilities can

be far. Nevertheless, effort should be made to apply shoot ^{15}N-labeling techniques to the field to evaluate various factors that might influence rhizodeposition under field conditions—for example, water stress and nutrient availability, and factors that affect plant-microbial interactions (e.g., mycorrhizal inoculants). In addition, evaluating differences in root biomass and rhizodeposition patterns among crops and cultivars that exhibit differing root morphology and distribution should be applied to field settings. A recent study using ^{13}C labeling in field microplots (Kong and Six, 2010) highlights the promise for dual-isotope labeling even in the field.

Combining stable isotope labeling techniques that enrich the entire plant with ^{15}N and/or ^{13}C with stable isotope probing (SIP) of nucleic acids can be used to identify specific microorganisms that are actively involved in crop residue decomposition (España et al., 2011a; España et al., 2011b). Currently these methods require highly enriched organic material (>90 atom% ^{15}N) for detection (Cadisch et al., 2005), which currently is not achieved in the case of rhizodeposits. However, as labeling and SIP methods improve, these limitations may be overcome, and in future may be applied to further our understanding of the influence of plant-soil-microbial interactions on C and N cycles.

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APPENDIX A. SOIL ATMOSPHERIC CONDITIONS IN POTS CONNECTED TO THE CLOSED LOOP $^{15}N_2$ LABELING SYSTEM

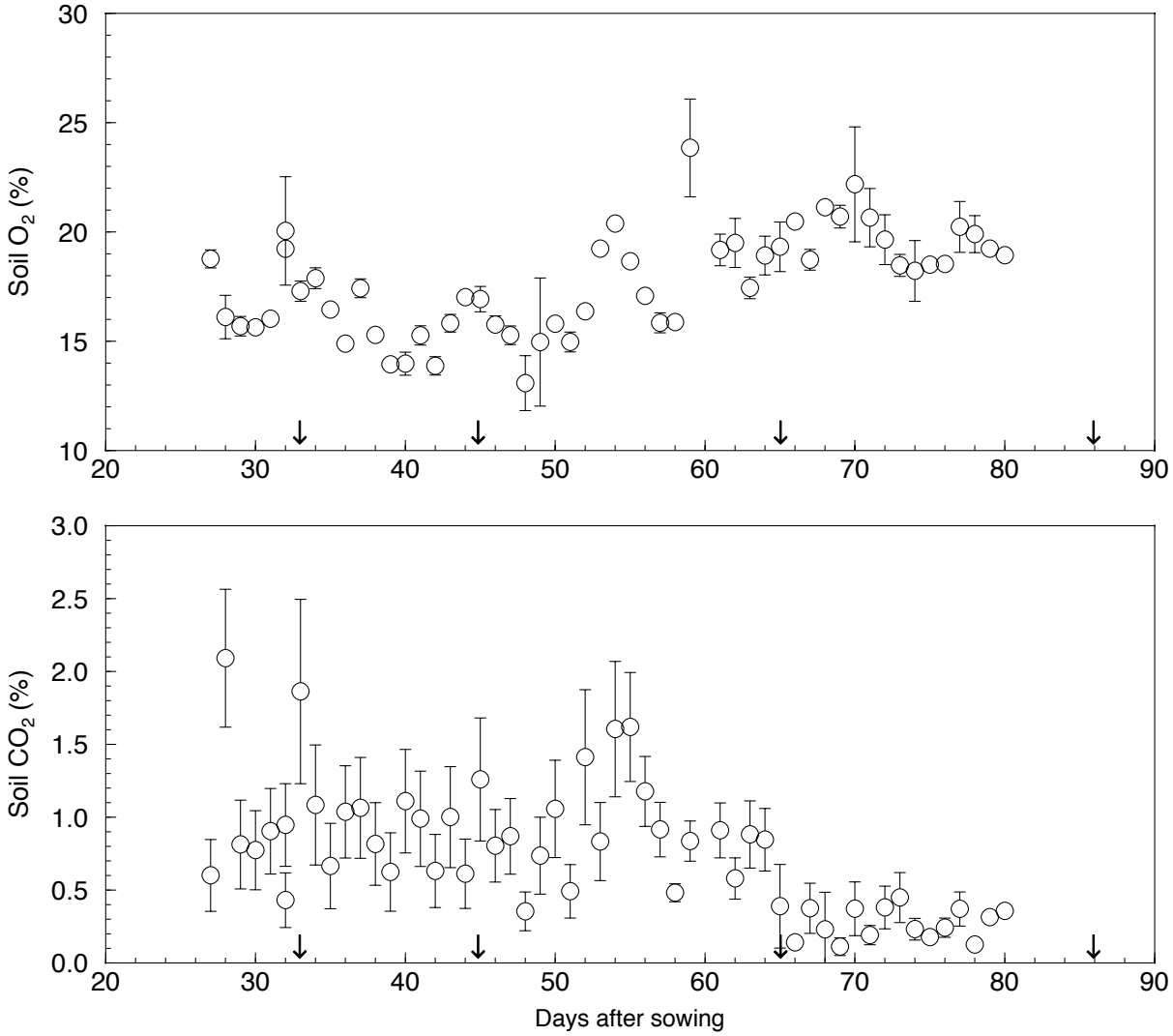

Fig. A.1. Soil atmosphere O_2 and CO_2 over the course of the $^{15}N_2$ labeling experiment outlined in Chapter 3. The arrows correspond to field pea harvest dates: 13 leaves unfolded (33 DAS), flowering (45 DAS), pod filling (65 DAS), and maturity (86 DAS). Markers represent means and error bars indicate standard deviations; the number of samples analyzed decreased over time as plants were harvested: n=24 until vegetative harvest; n=18, from vegetative to flowering; n=12 from flowering to pod filling; and n=6 from pod filling to maturity.

APPENDIX B. NITROGEN UPTAKE IN FIELD PEA USED TO DETERMINE ^{15}N-UREA APPLICATION RATES FOR COTTON-WICK LABELING

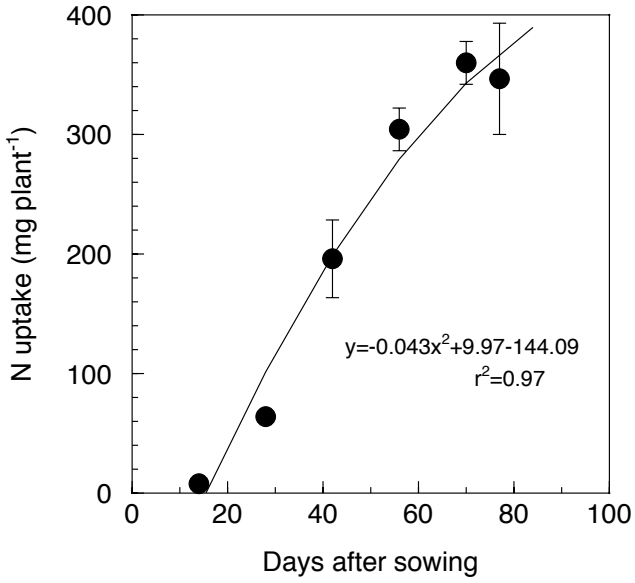

Fig. B.1. Mean N uptake (n=3) of field pea grown and harvested at various times over the course of plant growth in a greenhouse as part of a preliminary experiment to the study outlined in Chapter 4 for the purpose of estimating the rate of ^{15}N-urea application to the stems of field pea plants that were ^{15}N-labeled using the cotton-wick technique. Error bars represent standard deviations.